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CYTOKINE ENHANCED IMMUNOTHERAPY FOR BRAIN TUMORS

Field of the Invention

This invention is in the field of treating cancer by administering a combination of systemic and local immunotherapy using cytokines.

Rights of the Federal Government

The United States government has certain rights in this invention by virtue of a grant by the National Cooperative Drug Discovery Group UO1-CA52857 of the National Cancer Institute of the National Institutes of Health, Bethesda, Maryland.

Background of the Invention

One-third of all individuals in the United States alone will develop cancer. Although the five year survival rate has risen dramatically to nearly fifty percent as a result of progress in early diagnosis and therapy, cancer still remains second only to cardiac disease as a cause of death in the United States. Twenty percent of Americans die from cancer, half due to lung, breast, and colon-rectal cancer.

Designing effective treatments for patients with cancer has represented a major challenge. The current regimen of surgical resection, external beam radiation therapy, and/or systemic chemotherapy has been partially successful in some kinds of malignancies, but has not produced satisfactory results in others. In some malignancies, such as brain malignancies, this regimen produces a median survival of less than one year. For example, 90% of resected malignant gliomas recur within two centimeters of the original tumor site within one year.

Though effective in some kinds of cancers, the use of systemic chemotherapy has had minor success in the treatment of cancer of the colon-rectum, esophagus, liver, pancreas, and kidney. A major problem with systemic chemotherapy for the treatment of these types of cancer is that the systemic doses required to achieve control of tumor growth frequently result in unacceptable systemic toxicity. Efforts to improve delivery of

chemotherapeutic agents to the tumor site have resulted in advances in organ-directed chemotherapy, as by continuous systemic infusion, for example. However, continuous infusions of anticancer drugs generally have not shown a clear benefit over pulse or short-term infusions. Implantable elastomer access ports with self-sealing silicone diaphragms have also been tried for continuous infusion, but extravasation remains a problem. Portable infusion pumps are now available as delivery devices and are being evaluated for efficacy. (See Harrison's Principles of Internal Medicine, pp. 431-446, Braunwald, E., et al., ed., McGraw-Hill Book Co. (1987), for a general review). Controlled release biocompatible polymers have been used successfully for local drug delivery and have been utilized for contraception, insulin therapy, glaucoma treatment, asthma therapy, prevention of dental related disorders, and certain types of cancer chemotherapy. (Langer, R., and Wise, D., eds, Medical Applications of Controlled Release, Vol. I and II, Boca Raton, CRC Press (1986)).

In the brain, the design and development of effective anti-tumor agents for treatment of patients with malignant neoplasms of the central nervous system have been influenced by two major factors: 1) the blood-brain barrier provides an anatomic obstruction, limiting access of drugs to these tumors; and 2) the drugs given at high systemic levels are generally cytotoxic. Efforts to improve drug delivery to the tumor bed in the brain have included transient osmotic disruption of the blood brain barrier, cerebrospinal fluid perfusion, and direct infusion into a brain tumor using catheters. Each technique has had significant limitations. Disruption of the blood brain barrier increased the uptake of hydrophilic substances into normal brain, but did not significantly increase substance transfer into the tumor. Only small fractions of agents administered into the cerebrospinal fluid actually penetrated into the brain parenchyma. Drugs that have been used to treat tumors by infusion have been inadequate, did not diffuse an adequate distance from the site of infusion, or could not be maintained at a sufficient concentration to allow a sustained diffusion gradient. The use of catheters has been complicated by high rates of infection, obstruction, and malfunction

due to clogging. See Tomita, T., "Interstitial chemotherapy for brain tumors: review", *J. Neuro-Oncology*, 10:57-74 (1991).

Recent advances in the understanding of the immune system and advances in defining T cell antigens on tumor cells have shown promising results in treating tumors with immunotherapy. Several of these new approaches are aimed at augmenting weak host immune responses to tumor antigens and include the use of antibodies, cellular immunotherapy, and cytokines. The use of antibodies is difficult because it requires that the specificity of the antibody be such that it does not significantly bind to non-tumor cells. There are few truly tumor-specific antigens to select when an antibody-based immunotherapy approach is designed. Cellular immunotherapy involves the transfer of cultured immune cells that have anti-tumor reactivity into a tumor-bearing host. Adoptive therapy with autologous lymphokine activated killer (LAK) cells has yielded impressive results but only in the presence of cytokines or chemotherapeutic drugs. The use of cytokines administered directly to cells to enhance immune responses has also been shown to be successful. See Abbas, et al., "Immunity to Tumors", page 372, Cellular and Molecular Immunology, 2nd Ed., W.B. Saunders Company (1994). However, the effective administration of cytokines suffers from the same obstacles discussed above in reference to administration of chemotherapeutic agents in that high doses of systemic administration can be toxic and providing continuous release by local administration can be problematic.

It is therefore an object of the present invention to provide a composition and method of use thereof which provides for an enhanced immune response against tumors, especially brain tumors, improves therapeutic efficacy and diminishes potential toxicity.

It is a further object of the present invention to provide a composition and method of use for the treatment of tumors, especially brain tumors, which establishes long term memory capable of generating potent anti-tumor responses against multiple subsequent tumor challenges.

Summary of the Invention

A therapy for treatment of patients with cancer utilizing the combination of a cytokine in a pharmaceutical acceptable carrier for systemic administration and a cytokine in a pharmaceutical acceptable carrier for local administration is described. In the most preferred embodiment, brain tumors are treated with a cytokine such as granulocyte macrophage-colony stimulating factor (GM-CSF) administered systemically, most preferably in combination with tumor antigen such as replication incompetent tumor cells, and a cytokine such as interleukin-2 (IL-2) or IL-4 administered locally, most preferably in a vehicle providing release over a period of time, such as transduced cells, again most preferably replication incompetent tumor cells, or incorporated into microparticulate vehicles such as polymeric microspheres.

The examples demonstrate that systemic administration (vaccination) with GM-CSF transduced tumor cells or microencapsulated GM-CSF protects against growth of intracranial melanoma. The examples also demonstrate that local intracranial delivery of IL-2 transduced tumor cells or microencapsulated IL-2 generates immediate anti-tumor responses within the central nervous system as well as long term memory capable of generating potent anti-tumor responses against multiple subsequent tumor challenges, including challenges outside the central nervous system. The examples further demonstrate that combination immunotherapy using systemic vaccination with GM-CSF transductants and local intracranial administration of IL-2 transductants produces an anti-tumor effect that is significantly enhanced as compared to treatment with either treatment alone.

Brief Description of the Drawings

Figure 1 is a graph showing percent survival over time in days of experimental animal models of intracranial melanoma having received stereotactic intracranial injections of wild type B16-F10 melanoma cells in doses of 100,000 (circles), 10,000 (squares), 1,000 (triangles), and 100 (solid squares), and saline with no cells (solid circles).

Figure 2 is a graph showing percent survival over time in days of mice after systemic vaccination with a single subcutaneous injection of 10^6 irradiated B16-F10 cells engineered by gene transfer to secrete GM-CSF (solid squares), medium (circles) or 10^6 irradiated B16-F10 wild type non-cytokine producing cells (squares), challenged 14 days later with an injection of 10^2 non-irradiated wild type B16-F10 melanoma cells in the brain, ($p<0.001$).

Figure 3 is a graph showing percent survival over time in days of mice treated with a single intracranial injection of 10^5 irradiated B16-F10 cells engineered by gene transfer to secrete IL-2 (solid squares), medium (circles) or 10^5 irradiated wild type B16-F10 non-cytokine producing cells (squares), challenged at the same time by stereotactic intracranial co-injections of 10^2 non-irradiated wild type B16-F10 cells, ($p<0.001$).

Figure 4 is a graph showing percent survival over time in days of animals vaccinated with a subcutaneous injection of 10^6 irradiated GM-CSF producing B16-F10 melanoma cells followed two weeks later with 5×10^4 irradiated IL-2-secreting B16-F10 cells administered intracranially and simultaneously challenged with intracranial co-injections of 10^2 non-irradiated wild type B16-F10 cells (solid squares). Control animals received GM-CSF vaccine alone followed by intracranial tumor challenge with wild type B16-F10 cells (x marks), or vaccination with medium alone followed by intracranial IL-2 therapy and tumor challenge (circles). Additional controls received vaccination with medium followed by intracranial therapy with medium and intracranial tumor challenge (squares).

Detailed Description of the Invention

A therapy for treatment of tumors has been developed which relies on the combination of an initial systemic "priming" of the immune system, most preferably through the combination of administration of a cytokine such as GM-CSF and tumor antigen such as replication incompetent tumor cells along with local release at the tumor site (or site of resection following tumor removal) of a cytokine such as IL-2 which enhances the immune response against the tumor cells. Local release can be obtained using any of several

means, but a preferred method is using microparticles to release cytokine over a period of at least days or transduced cells, most preferably replication incompetent tumor cells which are transduced with the gene encoding the cytokine to be released. The latter is shown to release for at least five days after implantation. Microparticles can be designed to release for between hours and weeks or even months, as required.

The examples show the *in vivo* treatment of brain tumors using GM-CSF administered systemically, and IL-2 and IL-4 administered intracranially. However, a variety of cytokines can be used. Similar results are expected for treatment of other tumor types.

I. Therapeutic Compositions

a. Cytokines

i. Granulocyte-macrophage colony stimulating factor. Granulocyte-macrophage colony stimulating factor (GM-CSF) is a 22 kD glycoprotein made by activated T cells and by activated mononuclear phagocytes, vascular endothelial cells, and fibroblasts. GM-CSF has been shown to prime systemic immune responses *via* stimulation of bone marrow derived antigen presenting cells. See Inaba, K., et al., *J. Exp. Med.*, 175:1157-67 (1992); Inaba, K., et al., *J. Exp. Med.*, 176:1693-702 (1992); Steinman, R., *Annual Rev. Immunology*, 9:271-81 (1991). *In vivo* studies with GM-CSF-transduced tumor cells reveal that local release of this cytokine results in the generation of CD4+ and CD8+ tumor-specific T lymphocytes and systemic protection from tumor challenge. Dranoff, G., et al., *Proc. Natl. Acad. Sci. U.S.A.*, 90:3539-43 (1993).

ii. Interleukin-2. Interleukin-2 (IL-2) is produced by CD4+ T cells, and in lesser quantities by CD8+ T cells. Secreted IL-2 is a 14 to 17 kD glycoprotein encoded by a single gene on chromosome 4 in humans. IL-2 acts on the same cells that produce it, i.e., it functions as an autocrine growth factor. IL-2 also acts on other T lymphocytes, including both CD4+ and CD8+ cells. IL-2 induces a local inflammatory response leading to activation of both helper and cytotoxic subsets of T cells. IL-2 also stimulates the growth of natural killer cells and enhances their cytolytic

function. *In vivo* studies with IL-2-secreting tumor cells demonstrate that powerful local and systemic antitumor immune responses are generated leading to the destruction of the transduced tumors in the flank. Fearon, E. R., et al., *Cell*, 60:397-403 (1990); Gansbacher, B., et al., *J. Exp. Med.*, 172:1217-24 (1990).

iii. Tumor necrosis factor. Tumor necrosis factor (TNF) was originally identified as a mediator of tumor necrosis present in the serum of animals exposed to bacterial lipopolysaccharide (LPS) such as endotoxin. The major endogenous source of TNF is the LPS-activated mononuclear phagocyte, although antigen-stimulated T cells, activated natural killer cells, and activated mast cells can also secrete this protein. In the mononuclear phagocyte, TNF is initially synthesized as a nonglycosylated transmembrane protein of approximately 25 kD. TNF has potent anti-tumor effects *in vitro*, although clinical trials of TNF in advanced cancer patients have been discontinued due to toxicity. TNF-α has a diverse range of biological properties including inducing expression of a number of cytokines such as interleukin-6, interleukin-8, GM-CSF, and granulocye-colony stimulating factor, as well as causing hemorrhagic necrosis in established tumors. TNF has been reported to generate tumor suppression after tumor cell-targeted TNF-α gene transfer. Blankenstein, T., et al., *J. Exp. Med.*, 173:1047-52 (1991).

iv. Interleukin-4. Interleukin-4 (IL-4) is a helper T cell-derived cytokine of approximately 20 kD which stimulates the proliferation of mouse B cells in the presence of anti-Ig antibody (an analog of antigen) and causes enlargement of resting B cells as well as increased expression of class II MHC molecules. The principal endogenous source of IL-4 is from CD4+ T lymphocytes. Activated mast cells and basophils, as well as some CD8+ T cells, are also capable of producing IL-4. IL-4 delivered intracranially displays antitumor activity analogous to observations following administration peripherally of IL-4 transduced tumors. Golumbek, P.T., et al., *Science*, 254:713-6 (1991); Yu, J.S., et al., *Cancer Res.*, 53:3125-8 (1993).

v. Gamma interferon. Gamma interferon (IFN-γ) is a homodimeric glycoprotein containing approximately 21 to 24 kD subunits. IFN-γ is produced by some CD4+ helper T cells and nearly all CD8+ T cells. Transcription is directly initiated as a consequence of antigen activation and is enhanced by IL-2 and interleukin-12. IFN-γ is also produced by natural killer cells. IFN-γ acts as a potent activator of mononuclear phagocytes, acts directly on T and B lymphocytes to promote their differentiation and acts to stimulate the cytolytic activity of natural killer cells. IFN-γ transduced non-immunogenic sarcoma has been reported to elicit CD8+ T cells against wild type tumor cells. Restifo, N., et al., *J. Exp. Med.*, 175:1423-28 (1992).

vi. Interleukin-3. Interleukin-3 (IL-3), also known as multilineage colony-stimulating factor, is a 20 to 26 kD product of CD4+ T cells that acts on the most immature marrow progenitors and promotes the expansion of cells that differentiate into all known mature cell types. IL-3 has been reported to enhance development of tumor reactive cytotoxic T cells by a CD4-dependent mechanism. Pulaski, B. A., et al., *Cancer Res.*, 53:2112-57 (1993).

vii. Interleukin-6. Interleukin-6 (IL-6) is a cytokine of approximately 26 kD that is synthesized by mononuclear phagocytes, vascular endothelial cells, fibroblasts, and other cells in response to IL-1 and, to a lesser extent, TNF. It is also made by some activated T cells. IL-6 transfected into Lewis lung carcinoma tumor cells has been reported to suppress the malignant phenotype and to confer immunotherapeutic competence against parental metastatic cells. Porgador, A., *Cancer Res.*, 52:3679-87 (1992).

viii. Interleukin-7. Interleukin-7 (IL-7) is a cytokine secreted by marrow stromal cells that acts on hematopoietic progenitors committed to the B lymphocyte lineage. IL-7 has been reported to induce CD42+ T cell dependent tumor rejection. Hock, H., et al., *J. Exp. Med.*, 174:1291-99 (1991).

ix. Granulocyte-colony stimulating factor. Granulocyte-colony stimulating factor (G-CSF) is made by the same cells that make GM-CSF. The secreted polypeptide is approximately 19 kD. G-CSF gene transfer has been reported to suppress tumorgenicity of murine adenocarcinoma. Colombo, M., et al., Cancer Res., 52:4853-57 (1991).

x. Other cytokines. Other cytokines are known in the art to have an anti-tumor effect and can be used in the pharmaceutical compositions described herein. Moreover, since cytokines are known to have an effect on other cytokines, one can administer the cytokine which elicits one of the cytokines described above, or directly administer one of the cytokines which is elicited. Additional cytokines are known to those skilled in the art and are described in Abbas, et al., "Cytokines", chapter 12, pp. 239-61, Cellular and Molecular Immunology, 2nd Ed., W.B. Saunders Company (1994).

b. Combinations with other biologically active compounds

The cytokines can also be administered in combination with other cytokines, antibodies, cultured immune cells that have anti-tumor reactivity including LAK cells, or chemotherapeutic agents, including radiation therapy. The active compounds can be incorporated into the same vehicle as the cytokines for administration, or administered in a separate vehicle.

i. Chemotherapeutic agents.

The cytokines can be used alone, or in combination with other chemotherapeutic agents. Examples of chemotherapeutic agents include cytotoxic agents such as paclitaxel, camptothecin, ternozolamide, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), adriamycin, platinum drugs such as cisplatin, differentiating agents such as butyrate derivatives, transforming growth factor such as factor-alpha-*Pseudomonas* exotoxin fusion protein, and antibodies to tumor antigens, especially glioma antigens, such as monoclonal antibody 81C6. These agents can be incorporated into polymeric matrices for delivery along with the cytokines. See, for example, Domb, et al., *Polym. Prepr.*, 32(2):219-220 (1991), reported incorporating the water soluble chemotherapeutic agents carboplatin, an analog of cisplatin, and 4-

hydroperoxycyclophosphamide into a biodegradable polymer matrix for treating tumors, with promising results in.

ii. Other pharmaceutically active compounds.

In variations of these embodiments, it may be desirable to include other pharmaceutically active compounds, such as steroidal antiinflammatories which are used to reduce swelling, antibiotics, antivirals, or anti-angiogenic compounds. For example, dexamethasone, a synthetic corticosteroid used systemically to control cerebral edema, has been incorporated into a non-biodegradable polymer matrix and tested in rat brain *in vitro* and *in vivo* for efficacy in reversing cerebral edema. Other compounds which can be included are preservatives, antioxidants, and fillers, coatings or bulking agents which may also be utilized to alter polymeric release rates.

c. Cytokine Formulations

The cytokines can be administered in a pharmaceutically acceptable carrier such as saline, phosphate buffered saline, cells transduced with a gene encoding the cytokine, microparticles, or other conventional vehicles.

i. Polymeric formulations

The cytokines can be encapsulated into a biocompatible polymeric matrix, most preferably biodegradable, for use in the treatment of solid tumors. The cytokine is preferably released by diffusion and/or degradation over a therapeutically effective time, usually eight hours to five years, preferably one week to one year. As used herein, microencapsulated includes incorporated onto or into or on microspheres, microparticles, or microcapsules. Microcapsules is used interchangeably with microspheres and microparticles, although it is understood that those skilled in the art of encapsulation will recognize the differences in formulation methods, release characteristics, and composition between these various modalities. The microspheres can be directly implanted or delivered in a physiologically compatible solution such as saline.

Biocompatible polymers can be categorized as biodegradable and non-biodegradable. Biodegradable polymers degrade *in vivo* as a function of

chemical composition, method of manufacture, and implant structure. Synthetic and natural polymers can be used although synthetic polymers may be preferred due to more uniform and reproducible degradation and other physical properties. Examples of synthetic polymers include polyanhydrides, polyhydroxyacids such as polylactic acid, polyglycolic acid and copolymers thereof, polyesters, polyamides, polyorthoesters, and some polyphosphazenes. Examples of naturally occurring polymers include proteins and polysaccharides such as collagen, hyaluronic acid, albumin and gelatin. The ideal polymer must also be strong, yet flexible enough so that it does not crumble or fragment during use.

Cytokines and optionally, other drugs or additives, can be encapsulated within, throughout, and/or on the surface of the implant. The cytokine is released by diffusion, degradation of the polymer, or a combination thereof. There are two general classes of biodegradable polymers: those degrading by bulk erosion and those degrading by surface erosion. The latter polymers are preferred where more linear release is required. The time of release can be manipulated by altering chemical composition; for example, by increasing the amount of an aromatic monomer such as p-carboxyphenoxy propane (CPP) which is copolymerized with a monomer such as sebacic acid (SA). A particularly preferred polymer is CPP-SA (20:80). Use of polyanhydrides in controlled delivery devices has been reported by Leong, et al., *J. Med. Biomed. Mater. Res.*, 19:941 (1985); *J. Med. Biomed. Mater. Res.*, 20:51 (1986); and Rosen, et al., *Biomaterials*, 4:131 (1983). U.S. Patents that describe the use of polyanhydrides for controlled delivery of substances include U.S. Patent 4,857,311 to Domb and Langer, U.S. Patent 4,888,176 to Langer, et al., and U.S. Patent 4,789,724 to Domb and Langer. Other polymers such as polylactic acid, polyglycolic acid, and copolymers thereof have been commercially available as suture materials for a number of years and can be readily formed into devices for drug delivery.

Non-biodegradable polymers remain intact *in vivo* for extended periods of time (years). Agents loaded into the non-biodegradable polymer

matrix are released by diffusion through the polymer's micropore lattice in a sustained and predictable fashion, which can be tailored to provide a rapid or a slower release rate by altering the percent cytokine loading, porosity of the matrix, and implant structure. Ethylene-vinyl acetate copolymer (EVAc) is an example of a nonbiodegradable polymer that has been used as a local delivery system for proteins and other macromolecules, as reported by Langer, R., and Folkman, J., *Nature (London)*, 263:797-799 (1976). Others include polyurethanes, polyacrylonitriles, and some polyphosphazenes.

In the preferred embodiment, only polymer and cytokines to be released are incorporated into the delivery device, although other biocompatible, preferably biodegradable or metabolizable, materials can be included for processing purposes as well as additional therapeutic agents.

Buffers, acids and bases are used to adjust the pH of the composition. Agents to increase the diffusion distance of agents released from the implanted polymer can also be included.

Fillers are water soluble or insoluble materials incorporated into the formulation to add bulk. Types of fillers include sugars, starches and celluloses. The amount of filler in the formulation will typically be in the range of between about 1 and about 90% by weight.

Spheronization enhancers facilitate the production of spherical implants. Substances such as zein, microcrystalline cellulose or microcrystalline cellulose co-processed with sodium carboxymethyl cellulose confer plasticity to the formulation as well as implant strength and integrity. During spheronization, extrudates that are rigid, but not plastic, result in the formation of dumbbell shaped implants and/or a high proportion of fines. Extrudates that are plastic, but not rigid, tend to agglomerate and form excessively large implants. A balance between rigidity and plasticity must be maintained. The percent of spheronization enhancer in a formulation depends on the other excipient characteristics and is typically in the range of 10 to 90% (w/w).

Disintegrants are substances which, in the presence of liquid, promote the disruption of the implants. The function of the disintegrant is to

counteract or neutralize the effect of any binding materials used in the formulation. The mechanism of disintegration involves, in large part, moisture absorption and swelling by an insoluble material. Examples of disintegrants include croscarmellose sodium and crospovidone which are typically incorporated into implants in the range of 1 to 20% of total implant weight. In many cases, soluble fillers such as sugars (mannitol and lactose) can also be added to facilitate disintegration of the implants.

Surfactants may be necessary in implant formulations to enhance wettability of poorly soluble or hydrophobic materials. Surfactants such as polysorbates or sodium lauryl sulfate are, if necessary, used in low concentrations, generally less than 5%.

Binders are adhesive materials that are incorporated in implant formulations to bind powders and maintain implant integrity. Binders may be added as dry powder or as solution. Sugars and natural and synthetic polymers may act as binders. Materials added specifically as binders are generally included in the range of about 0.5 to 15% w/w of the implant formulation. Certain materials, such as microcrystalline cellulose, also used as a spheronization enhancer, also have additional binding properties.

Various coatings can be applied to modify the properties of the implants. Three types of coatings are seal, gloss and enteric. The seal coat prevents excess moisture uptake by the implants during the application of aqueous based enteric coatings. The gloss coat improves the handling of the finished product. Water-soluble materials such as hydroxypropyl cellulose can be used to seal coat and gloss coat implants. The seal coat and gloss coat are generally sprayed onto the implants until an increase in weight between about 0.5% and about 5%, preferably about 1% for seal coat and about 3% for a gloss coat, has been obtained.

Enteric coatings consist of polymers which are insoluble in the low pH (less than 3.0) of the stomach, but are soluble in the elevated pH (greater than 4.0) of the small intestine. Polymers such as Eudragit®, RohmTech, Inc., Malden, MA, and Aquateric®, FMC Corp., Philadelphia, PA, can be used and are layered as thin membranes onto the implants from aqueous

solution or suspension. The enteric coat is generally sprayed to a weight increase of about one to about 30%, preferably about 10 to about 15%, and can contain coating adjuvants such as plasticizers, surfactants, separating agents that reduce the tackiness of the implants during coating, and coating permeability adjusters. Other types of coatings having various dissolution or erosion properties can be used to further modify implant behavior. Such coatings are readily known to one of ordinary skill in the art.

Controlled release devices are typically prepared in one of several ways. For example, the polymer can be melted, mixed with the substance to be delivered, and then solidified by cooling. Such melt fabrication processes require polymers having a melting point that is below the temperature at which the substance to be delivered and polymer degrade or become reactive. Alternatively, the device can be prepared by solvent casting, where the polymer is dissolved in a solvent, and the substance to be delivered dissolved or dispersed in the polymer solution. The solvent is then evaporated, leaving the substance in the polymeric matrix. Solvent casting requires that the polymer be soluble in organic solvents and that the agents to be encapsulated be soluble or dispersible in the solvent. Similar devices can be made by phase separation or emulsification or even spray drying techniques. In still other methods, a powder of the polymer is mixed with the cytokine and then compressed to form an implant.

Methods of producing implants also include granulation, extrusion, and spheronization. A dry powder blend is produced including the desired excipients and microspheres. The dry powder is granulated with water or other non-solvents for microspheres such as oils and passed through an extruder forming "strings" or "fibers" of wet massed material as it passes through the extruder screen. The extrudate strings are placed in a spheronizer which forms spherical particles by breakage of the strings and repeated contact between the particles, the spheronizer walls and the rotating spheronizer base plate. The implants are dried and screened to remove aggregates and fines. These methods can be used to make micro-implants

(microparticles, microspheres, and microcapsules encapsulating cytokines to be released), slabs or sheets, films, tubes, and other structures.

ii. Cytokine secreting Genetically engineered cells

Cytokine secreting cells can be genetically engineered by methods known in the art to deliver cytokines systemically or locally. Cells can either be transformed or transduced, with bacterial or viral DNA vectors, respectively. In a preferred embodiment the cells are tumor cells. To prevent replication and preserve cytokine production, the transduced cells can be irradiated prior to *in vivo* injection. The advantage of the tumor cells is that the patient is exposed to antigen in combination with cytokine stimulating the immune response to the cytokine. Examples of the preparation of tumor cells engineered to secrete IL-4 are described in Golumbek, P. T., et al., *Science*, 254:713-6 (1991) and Yu, J. S., et al., *Cancer Res.* 53:3125-8 (1993). IL-2 gene transfer into tumor cells is described in Gansbacher, B., et al., *J. Exp. Med.*, 172:1217-24 (1990). Tumor cells engineered to secrete GM-CSF are described in Dranoff, G., et al., *Proc. Natl. Acad. Sci. U.S.A.*, 90:3539-43 (1993). TNF gene transfer is described in Blankenstein, T., et al., *J. Exp. Med.*, 73:1047-52 (1991). IL-6 transfection into lung carcinoma tumor cells is described in Progador, A, et al., *Cancer Res.*, 52:3679-87 (1992). γIFN cDNA transduced into non-immunogenic sarcoma is described in Restifo, N., *J. Exp. Med.*, 175:1423-28 (1992). G-CSF gene transfer is described in Colombo, M., et al., Cancer Res., 52:4853-57 (1991). Other cytokines including IL-3 and IL-7 can be secreted from cells by applying the methods used for the cytokines described above or by the general procedures for genetically modifying nonimmunogenic murine fibrosarcoma described in Karp, S. E., et al., *J. Immunol.*, 150:896-908 (1993) and Jaffee, E. et al., *J. Immunotherapy*, in press (1995). The publications cited above describe methods of genetically engineering cells to secrete cytokines. Additionally, cancer vaccines expressing two or more cytokines from engineered vectors containing genes encoding two different cytokines or by sequential recombinant retrovirus-mediated genetic transductions can be prepared. Suitable pharmaceutical vehicles are known to those skilled in the art.

Inducers can also be used to produce cells which secrete cytokines. Inducers may be used alone in some cases or in combination with transforming agents, and include tumor necrosis factor (TNF), endotoxin, and other agents known to those skilled in the art. In general, the cultured cells are exposed to an amount effective to activate the cells, as determined by cytokine expression, immunoglobulin secretion, and/or other indicators such as proliferation or alteration of cell surface properties or markers. In some cases, cells are initially exposed to a small amount to "prime" the cells, then to a subsequent dose to elicit greater activation of the cells.

Cells can be administered in medium or washed and administered in saline. Alternatively, cells can be encapsulated in a polymeric matrix and administered.

II. Administration to Patients

The cytokines described herein or their functionally equivalent derivatives can be administered alone or in combination with, either before, simultaneously, or subsequent to, treatment using other chemotherapeutic or radiation therapy or surgery. A preferred embodiment is systemic administration of a first cytokine such as GM-CSF, most preferably in combination with tumor antigen in the form of replication incompetent tumor cells, followed by local administration during surgery or by injection of either a biocompatible polymeric matrix loaded with the selected cytokine or cytokine secreting cells, using dosages determined as described herein. Local administration can be at a site adjacent to the tumor to be removed, or in the tumor, or in the place where the tumor was removed. The dosages for functionally equivalent derivatives can be extrapolated from the *in vivo* data. An effective amount is an amount sufficient to induce an anti-tumor effect as measured either by a longer survival time or decrease in tumor size.

Local administration can also be accomplished using an infusion pump, for example, of the type used for delivering insulin or chemotherapeutic agents to specific organs or tumors.

In the preferred method of administration, the polymeric implants or cytokine secreting cells are implanted at the site of a tumor, two weeks after

systemic administration of the polymeric implants or cytokine secreting cells, for example, by subcutaneous injection at a distant site. If biodegradable polymers are used, preferably they are less than about 100 to 200 microns in diameter and injected by means of a catheter or syringe; it is not necessary to remove the implant following release of the immunotherapeutic agent.

The cytokines can also be combined with other therapeutic modalities, including radiotherapy, chemotherapeutic agents administered systemically or locally, and other immunotherapy.

The cytokines are preferably administered to brain cancer patients. However, the cytokine combination therapy can also be applied to all cancer patients including those having breast cancer, lung cancer, and colon cancer. The treatment will provide an immediate response at the site of the local administration as well as provide long term memory protection against cancer at the site of local administration and at a site distant from the site of local administration. The immunotherapy described herein is therefore useful in preventing or treating metastases.

III. Examples.

The present invention will be further understood by reference to the following non-limiting examples.

Example 1: Systemic vaccination with GM-CSF-transduced tumor cells

Tumor cell lines and animals. B16-F10 melanoma cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum and penicillin/streptomycin. Animals used for all experiments were 6 to 12 week old C57BL/6 female mice obtained from Harlan (Indianapolis, IN). B16-F10 melanoma cells were transduced with murine GM-CSF gene by using the replication-defective MFG retroviral vector as previously described in Dranoff, G., et al., *Proc. Natl. Acad. Sci. U.S.A.*, 90:3539-43 (1993). The amount of cytokine produced by the transformed tumor cells was quantified routinely by a standard ELISA technique (Endogen, Cambridge, MA). Cultured tumor monolayers were harvested with trypsin and resuspended in DMEM before injection. Tumor cells were exposed to 5000 rads from a ^{137}cesium source (Gammacell Model

#62 irradiator, Nordin International, Inc., Kanata, Ontario, Canada) discharging 1378 rads per minute, immediately before injection in order to render them replication incompetent.

Technique for intracranial cell injections. Mice were anesthetized with an intraperitoneal injection of 0.1 ml of a stock solution containing ketamine hydrochloride 25 mg/ml, xylazine 2.5 mg/ml, and 14.25% ethyl alcohol diluted 1:3 in 0.9% NaCl solution. For stereotactic intracranial injections of tumor cells, the surgical site was shaved and prepared with 70% ethyl alcohol and prepodyne solution. After a midline incision, a 1 mm burr hole center 2 mm posterior to the coronal suture and 2 mm lateral to the sagittal suture was made. Animals were then placed in a stereotactic frame and cells were delivered by a 26 gauge needle to a depth of 3 mm over a period of 3 minutes. The total volume of injected cells was 10 μl. The needle was removed, the site irrigated with sterile 0.9% NaCl solution, and the skin was sutured closed with 4.0 vicryl.

Development of an intracranial B16-F10 melanoma model in C57BL/6 mice. To assess the intracranial growth characteristics of B16-F10 melanoma, animals were divided into five groups of at least eight animals and received stereotactic intracranial injections of either 10^2, 10^3, 10^4, or 10^5 wild type B16-F10 melanoma cells into the left parietal region by the method described above. Control animals received similar intracranial injections of 0.9% NaCl solution. Animals were assessed daily for survival. For histologic analysis, brains were removed at the time of death and fixed in 10% formalin for at least 5 days, sectioned, embedded in paraffin, and stained with hematoxylin and eosin.

All animals receiving the highest dose of B16-F10 (10^5 cells) died within 15 days of treatment, with a median survival of 12 days. The results are shown in Figure 1. At doses of 10^3 and 10^4 cells the median survival was 20 and 16 days, respectively. Intracranial does of 10^2 cells were uniformly fatal (median survival 20 days, range 16 to 21 days). These kinetics emphasize the highly tumorigenic, poorly immunogenic character of

B16-F10. All control animals (treated with stereotactic injections of 0.9% NaCl) were alive at 120 days.

Histologic analysis of the brains revealed that all animals treated with intracranial wild type B16-F10 developed large, solid tumor masses at the injection in the parietal region. Metastatic deposits of tumor were occasionally observed in the brain parenchyma distant from the injection site, especially in those animals receiving large tumor inoculations. Tumor deposits were also observed in the subarachnoid space surrounding the spinal cord, suggesting that cell migration occurred along cerebrospinal fluid pathways. No tumor was apparent outside the craniospinal axis. This new model has proven to be highly reliable and reproducible. With intracranial doses of 10^2 tumor cells, animals died consistently over a 3 to 4 day period within 20 days of injection.

Vaccination studies. Cytokine-secreting B16-F10 melanoma cells were evaluated for their vaccination properties and ability to protect against intracranial challenge with wild type B16-F10 melanoma. For these studies, animals were treated with a single subcutaneous flank injection of 10^6 irradiated GM-CSF secreting B16-F10 cells using a tuberculin syringe with a 27 gauge needle. The total amount of GM-CSF secreted by the transduced cells *in vitro* prior to injection was between 50 and 60 ng per 10^6 cells per day. To prevent replication and preserve cytokine production, the transduced cells were irradiated prior to *in vivo* injection. Previous studies had shown that B16-F10 melanoma cells exposed to 5000 rads are rendered replication incompetent, but maintain their metabolic viability and continue to secrete cytokine *in vivo* for up to 5 days, Jaffee, E. et al., "Use of murine models of cytokine-secreting tumor vaccines to study feasibility and toxicity issues critical to designing clinical trials", *J. Immunotherapy*, in press (1995). The experiments were conducted with two control groups: one group received flank injections of 10^6 irradiated wild type (non-cytokine-producing) B16 cells and the other was treated with a subcutaneous injection of either cell growth medium or 0.9% NaCl. There were at least 10 animals per group for all experiments. After 2 weeks all animals were challenged with stereotactic

intracranial injections of 10^2 non-irradiated wild type B16-F10 melanoma cells by the method outlined above. Animals were assessed for survival.

Statistical analysis. For all efficacy studies, survival was plotted using a Kaplan-Meier survival analysis and statistical significance was determined by the Kruskal-Wallis nonparametric analysis of variance as described in Zar, J., eds., Biostatistical Analysis, Englewood Cliffs, New Jersey, Prentice-Hall, Inc., pp. 140-50 (1984). Percentages of long term survivors were analyzed by comparison of two proportions.

Results

Figure 2 shows the results of the systemic vaccination studies performed on animals receiving single subcutaneous injections of 10^6 irradiated B16-F10 cells engineered by gene transfer to secrete GM-CSF. GM-CSF secreting cells administered in this manner were found to protect against intracranial challenge with the wild type tumor. Median survival for animals treated with the GM-CSF vaccine and then challenged 2 weeks later with wild type B16 in the brain was 27 days, compared to 19 and 17 days for control animals treated with a vaccine of irradiated, wild type cells (which did not secrete GM-CSF) or medium, respectively ($p < 0.0001$). These results have been replicated 4 times, each with similar and statistically significant results.

Example 2: Systemic vaccination with microencapsulated GM-CSF

Thirty-two C57BL/6 mice were divided into four groups of eight and given flank vaccinations with either: 1) saline, 2) 2500 ng bolus GM-CSF and 1 x 10^6 irradiated B16 melanoma cells, 3) 2500 ng GM-CSF microspheres and 1 x 10^6 irradiated melanoma cells, or 4) 1 x 10^6 irradiated melanoma cells transduced to produce GM-CSF. Fourteen days later all animals received a challenge of B16 melanoma in the left frontal lobe.

Results

The median survival of the GM-CSF microsphere group and the transduced GM-CSF secreting group was 27.5 days each. These survival periods are significantly prolonged compared to the control animals receiving saline which survived 16.5 days, ($p < 0.001$). The GM-CSF bolus group

survived 15.5 days, therefore having no effect on survival. No GM-CSF treated animal showed any sign of toxicity from the vaccination.

This study demonstrates that a significant anti-tumor immune response is generated using a systemic vaccination with GM-CSF microspheres. This response is comparable to that generated by GM-CSF transduced tumor cells and represents a practical method for delivering GM-CSF in treatments employing GM-CSF immunotherapy.

Example 3: Local intracranial delivery of IL-2-transduced tumor cells

Animals received intracranial stereotactic co-injections of either 10^3, 10^4, 10^5, or 10^6 irradiated cytokine-producing B16-F10 melanoma cells, which produce 80 ng of IL-2 per 10^6 cells every twenty-four hours as determined by ELISA. Control animals received co-injections of irradiated non-IL-2 producing wild type B16-F10 at the equivalent doses of 10^3, 10^4, 10^5, or 10^6 cells. Simultaneously, all animals were challenged with stereotactic intracranial injections of 10^2 live non-irradiated B16-F10 melanoma cells. For these experiments, there were at least 10 animals per group.

Results

IL-2 transduced cells were found to be highly effective in treating intracranial tumor when they were delivered directly to the brain. Initial studies showed that the IL-2 effect was dose-dependent. The results are shown in Figure 3. Doses of 0.08 ng per day of IL-2 (10^3 IL-2 producing cells) showed no enhanced survival compared to controls, whereas a dose of 0.8 ng per day (10^4 IL-2 producing cells) showed a trend toward prolonged survival that did not reach statistical significance. When tested at a dose of 8.0 ng per day (10^5 IL-2 producing cells), a significant prolongation in survival was seen. While untreated control animals had died with intracranial tumor by day 22 (median survival, 20 days), median survival for the IL-2 group was 31 days with 33% of the mice living more than 80 days, ($p < 0.001$). Interestingly, several animals that died in the IL-2 treatment group were found to have no tumor in the brain; rather, tumor was observed surrounding the spinal cord suggesting that the protective effect of IL-2 was

localized to the site of cytokine delivery. At higher doses of IL-2 (80.0 ng per day), signs of IL-2 toxicity were observed including ataxia and death. These animals died before controls (median survival, 12 days). These studies have been replicated 7 times with similar doses of IL-2 transductants. In each experiment, there was a significant prolongation of survival in animals treated with intracranial IL-2 transductants as compared to controls.

On histologic examination at the time of death, inflammation was notably absent in the brain parenchyma of the treated animals. Lymphocytes were observed, however, in the cerebrospinal fluid spaces of the IL-2 treated animals.

Example 4: Intracranial delivery of microencapsulated interleukin-2 produces long term memory and protects against re-challenges

IL-2 was incorporated into biodegradable sustained release microspheres having an average diameter of 4 μm. These microspheres were stereotactically injected into the brains of mice to deliver 100 ng of IL-2 over a 7 day period. Safety testing showed no evidence of clinical or histologic toxicity from this dose over a 120 day period. To evaluate efficacy as a primary treatment, a lethal challenge of B16 melanoma was stereotactically co-injected into the left parietal lobe of 25 mice with either saline, empty microspheres, or the IL-2 containing microspheres. In a similar experiment, 9L glioma cells were co-injected into the left parietal lobe of rats.

To evaluate the potential brain vaccine effect of this therapy, a group of non tumor-bearing mice were co-injected intracranially with IL-2 microspheres and melanoma cells which had been irradiated to prevent replication. Thirty days later these animals and a group of control mice having no previous treatment were challenged intracranially with the lethal dose of live melanoma with no additional IL-2 therapy.

Results

Seventy-five percent of the IL-2 treated mice (6 of 8) were alive with no sign of tumor 90 days after initial treatment while median survival of untreated controls was 18 days. No animal survived more than 21 days. The empty microspheres had no effect on survival. In a similar mouse

melanoma experiment, no impact on survival from either a bolus injection of IL-2 to the tumor site or systemic IL-2 administration was observed. In the glioma experiments, microsphere delivery of IL-2 prolonged median survival in rats to 55 days compared to 25 days for the controls.

Previous IL-2 vaccination extended median survival by 45 percent, with 2 to 5 animals alive at 160 days after tumor challenge with all controls dead by 23 days.

These results indicate that brain interstitial immunotherapy with IL-2 delivered by microspheres is an effective and safe treatment against brain metastases and inhibits tumor recurrence. Additional efficacy results and release kinetics of IL-2 from microspheres are reported in Sills, A. K., Jr., et al., *Proceed. Intern. Symp. Control. Rel. Biomater.*, 23 (July, 1996), and Kalyanasundaram, S., et al., *Proceed. Intern. Symp. Control. Rel. Biomater.*, 23 (July, 1996), respectively.

Example 5: Intracranial delivery of IL-2 transduced cells produces long term memory and protects against re-challenges

A group of 11 C57BL/6 mice were initially treated with intracranial IL-2 and co-injected with a dose of B16 melanoma that was uniformly fatal in control animals. Unlike controls, all 11 animals who received intracranial IL-2 survived the initial tumor challenge in the brain and were rechallenged at the same site on day 70. Six of the 11 were rechallenged with wild-type melanoma cells. The other five received injections of media alone. Twelve additional mice, previously untreated, were similarly challenged with B16 melanoma in the brain.

Results

The melanoma challenge was fatal to all previously untreated mice, the median survival was 17 days. By contrast, the mice initially treated with IL-2 and rechallenged with tumor on day 70 had a significantly prolonged survival where the median survival was 36 days, with 3 of 6 surviving 70 days ($p=0.01$ versus positive control, Mann-Whitney Test). As expected, mice rechallenged with media all lived an additional 70 days.

These results demonstrate long-term memory in an immune response directed against intracranial melanoma useful in preventing metastatic tumor recurrence after resection or inhibiting growth of synchronous metastases by delivery of IL-2 transduced cells.

Example 6: Local intracranial delivery of IL-2 transduced cells protects against challenges outside the central nervous system

Twenty-four C57B16 mice were injected intracranially with irradiated, IL-2 secreting B16-F10 melanoma cells and 12 C57B16 mice were injected with medium, as controls. All animals simultaneously received a co-injection of wild type B16-F10 cells which were uniformly fatal to untreated mice. Nine of the twenty-four IL-2 treated animals survived 75 days. No controls survived more than 20 days. At day 75, the 9 surviving animals and 8 new control animals were challenged in the flank with 10,000 wild type B16-F10 melanoma cells.

Results

Only 1 of 9 long-term survivors developed a flank tumor; in contrast, 6 of 8 control mice developed a flank tumor, ($p=.024$). The 8 (of 9) long-term survivors who rejected the first flank challenge, along with 8 new control animals, were challenged a second time in the flank with a larger bolus of 50,000 wild type B16-F10 melanoma cells, 126 days after the first challenge. Seventeen days later, 8 of 8 control mice had developed tumors whereas only 2 of 8 of the long term survivors had a flank tumor ($p=0.01$).

This study demonstrates that localized IL-2 immunotherapy delivered intracranially not only generates an immediate anti-tumor response within the central nervous system, but also establishes long-term memory capable of generating potent anti-tumor responses against multiple subsequent tumor challenges outside the central nervous system.

Example 7: Local intracranial delivery of IL-4-transduced tumor cells

Poorly immunogenic B16-F10 melanoma cells were transfected with the IL-4 gene to deliver IL-4 at the site of a tumor in the central nervous system. The transfected B16-F10 cells were irradiated to prevent replication and injected intracranially in eight C57BL/6 mice along with non-irradiated,

non-transfected B16-F10 (wild type) melanoma cells. The expression of IL-4 receptors on B16-F10 melanoma cells was examined by means of fluorescence activated cell sorting (FACS).

Results

While animals treated with IL-4 had a median survival of 31 days, control animals that received only wild type cells had a median survival of 18 days (range 15 to 20 days; $p=0.002$).

FACS analysis of B16-F10 cells did not reveal the presence of the IL-4 receptor, suggesting that IL-4 does not interact with its receptor on tumor cells to exert the cytotoxic effect. The results obtained in these experiments indicate that high levels of IL-4 expressed locally stimulate a strong immunologic antitumor response which leads to significant prolongation of survival in mice with central nervous system tumors.

Example 8: Combination therapy: Systemic vaccination with GM-CSF transductants and local intracranial administration of IL-2 transductants

For combination experiments, all animals received a subcutaneous flank vaccination and were challenged after two weeks with the standard intracranial dose of 10^2 wild type melanoma cells co-injected with the specific intracranial treatment. The flank vaccination was with a single subcutaneous injection of 10^6 irradiated GM-CSF-producing cells and the intracranial treatment two weeks later was with 5×10^4 irradiated IL-2 producing cells injected into the brain. Given that a dose of 8.0 ng per day of IL-2 (10^5 cells), yielded a 33 percent long-term survivor rate and approached doses at which toxicity was observed, a lower dose of 4.0 ng per day of IL-2 (5×10^4 cells) was selected. Control animals received either the subcutaneous GM-CSF vaccine and intracranial treatment with medium, or subcutaneous vaccination with medium and intracranial treatment with 5×10^4 IL-2 producing cells. Additional controls received vaccinations of medium alone followed by intracranial treatment with medium along with an intracranial tumor challenge.

Results

When animals were treated with the GM-CSF-secreting cell vaccine in combination with intracranial IL-2-producing cells, a greatly enhanced response was achieved. The results are shown in Figure 4. Median survival was not yet reached at 100 days, with 58% of the mice still alive after combination therapy. This represents a significant advantage in survival over flank vaccination with GM-CSF-producing cells alone (median survival, 27 days, $p=0.018$), over local intracranial administration of IL-2-producing cells alone (median survival, 35 days, $p=0.01$), and over control (median survival, 17.5 days, $p<0.001$). The rate of 58% of long term survivors, those surviving more than 100 days, in the combination group exceeds the percentage in the GM-CSF flank vaccine group (16%, $p=0.08$), the intracranial IL-2 transductant group (8.3%, $p=0.03$), or the sum of the two, providing a greatly enhanced response. Both the mice receiving the GM-CSF vaccine alone and those treated with intracranial IL-2-secreting cells alone showed prolongation of survival compared to controls (IL-2, $p=0.0006$; GM-CSF, $p<0.001$), confirming the efficacy of single cytokine therapy in this model.

These results indicate that the GM-CSF vaccine generates a cohort of CD4+ and CD8+ T-cells specific for a tumor antigen, which activity of the cells is enhanced by the subsequent local IL-2 therapy at the tumor site. The results correspond with the theory that while the blood-brain barrier poses an obstacle to delivery of chemotherapeutic agents to the brain, it functions as an avenue of entry into the central nervous system for immunologically active cells. Wekerly, H., et al., *TINS,* 27:1-7 (1986).

The degree of inflammatory infiltrate in the brain parenchyma of animals treated with these cytokines was small, regardless of the mode of delivery. These findings are in contrast to studies of cytokine enhanced antitumor immune responses done in flank models, where a marked local inflammatory infiltrate was apparent. These results indicate that intrinsic immune response cells within the brain such as microglia may also be involved in the protective immune response.

Example 9: Systemic vaccination with GM-CSF transductants and local intracranial administration of IL-2 transductants produces long term memory protection against re-challenges

Genetically engineered non-replicating B16-F10 melanoma cells were used for systemic delivery of GM-CSF or intracranial delivery of IL-2. Forty-nine C57B16 mice were vaccinated in the flank with either GM-CSF producing B16-F10 cells or medium followed by intracranial delivery of IL-2 or medium 2 weeks later. Every animal received an intracranial co-injection of wild-type melanoma at a dose uniformly fatal to untreated animals. Initial survivors and a new control group were rechallenged with a second intracranial dose of wild-type B16-F10 melanoma 134 days after their first challenge.

Results

Animals receiving both flank GM-CSF and intracranial IL-2 had the longest survival, with median survival not yet reached at 130 days (7 of 13 alive). This is a significant improvement over either flank GM-CSF alone (median survival 27 days, $p=0.018$), intracranial IL-2 alone (32 days, $p=0.01$) or control (17.5 days, $p<0.001$). GM-CSF alone and IL-2 alone were significantly more effective than the controls, ($p<0.006$).

Seven animals who received combination therapy with GM-CSF and IL-2 survived more than 130 days. Five of the 7 mice rechallenged were alive 28 days later. All 8 controls died by day 21 ($p=0.018$). Furthermore, the antitumor effect persisted for greater than 4 months. These results demonstrate that use of cytokines can lead to immunologic memory in the brain.

Example 10: Interstitial delivery of IL-2 to the brain

Methods

An alternative approach to the control of metastatic brain tumors by using local immunotherapy with interstitial delivery of interleukin-2 (IL-2) as both a primary treatment for establishing tumors and a vaccine against subsequent recurrence is described. IL-2 was incorporated into biodegradable sustained release microspheres (average diameter 4 μm) which were stereotactically injected into the brains of mice to deliver 100 ng IL-2 over a

7 day period. Safety testing showed no evidence of clinical or histologic toxicity from this dose over a 120 day period. To evaluate efficacy as a primary treatment, a lethal challenge of B16 melanoma was stereotactically co-injected into the left parietal lobe of 25 mice with either: saline (controls), empty (no IL-2) microspheres, or the IL-2 containing microspheres.

Results

75% of the IL-2 treated animals (6/8) were alive with no sign of tumor 90 days after initial treatment while median survival of untreated controls was 18 days with no animal surviving more than 21 days. The empty microspheres had no effect on survival. In a similar mouse melanoma test, no impact was seen on survival from either bolus injection of IL-2 to the tumor site or systemic IL-2 administration, suggesting that the sustained local delivery of IL-2 in the brain provided by the microspheres is important for the continued stimulation of immune cells attracted to the tumor bed.

Example 11: Intracranial injections of IL-2 into the brain

Methods

To evaluate the potential brain vaccine effect of this therapy, a group of non tumor-bearing mice were co-injected intracranially with IL-2 microspheres and melanoma cells (see example 10) which had been irradiated to prevent replication. 30 days later these animals and a group of control mice (no previous treatment) were challenged intracranially with the lethal dose of live melanoma with no additional IL-2 therapy.

Results

Previous IL-2 vaccination extended median survival by 45% and generated 2/5 animals alive at 160 days after tumor challenge with all controls dead by 23 days. These results suggest that brain interstitial immunotherapy with IL-2 delivered by microspheres is an effective and safe treatment against brain metastases and may prevent tumor recurrence.

Example 12: Combination therapy with locally delivered IL-2 and chemotherapeutic agents

Methods

A combination therapy consisting of immunotherapy, using non-replicating genetically engineered tumor cells that produce IL-2, and local delivery of chemotherapy was tested to determine if the component therapies acted synergistically against an intracranial tumor challenge in a murine brain tumor model. B16-F10 melanoma cells were transduced with murine IL-2 using a replication-defective MFG retroviral vector. Chemotherapeutic agents, BCNU and carboplatin, were incorporated into controlled-release polymers. C57/B16 mice received intracranial challenge of wild-type tumor with and without non-replicating IL-2 producing tumor cells. On day 5, animals received polymer implants containing 4.0% BCNU, 1% carboplatin, or placebo.

Results

Animals receiving IL-2 and 1% carboplatin ($n=10$, median survival greater than 100 days, $p<0.01$) had significantly improved survival over animals receiving IL-2 ($n=10$, median survival 38 days) or 1% carboplatin alone ($n=$, median survival 23 days). Animals receiving IL-2 and 4.0% BCNU ($n=10$, median survival 44 days) had improved survival over animals receiving 4.0% BCNU ($n=10$, median survival 24 days) or IL-2 alone ($n=10$, median survival 38 days). The median survival for the control group ($n=10$) was 19 days. This example demonstrates that combination intracranial immunotherapy with IL-2 and local delivery of chemotherapy improves survival over either antitumor therapy alone. This effect was obtained even when utilizing low doses of chemotherapy (1% carboplatin and 4.0% BCNU).

Example 13: IL-2 inhibited tumor growth in murine model of hepatic melanoma metastasis

Methods

Useful immunotherapeutic strategies must demonstrate success in tumors within the liver, an organ with unique immunologic properties and a common site of metastases in humans. The anti-tumor effects of local or

paracrine IL-2 delivery was evaluated using the non-immunogenic B16-F10 melanoma implanted into the liver of C57B1/6 mice. Irradiated autologous tumor cells transfected with the IL-2 gene was used as the source of IL-2. Varying numbers of these cells (5×10^5 to 2×10^6) were admixed with 1×10^4 wild type B16-F10 melanoma cells and injected intrahepatically, and compared to cells alone, cells coinjected with non-transfected cells, or with IL-2 cells injected at a distant site (flank) (n=7/group). Presence or absence of tumor within the liver and tumor volumes were measured on day 21.

Results

Inhibition of tumor growth was seen in mice receiving locally delivered IL-2 at all doses. The number of mice without any tumor was significantly less in those receiving the highest IL-2 dose ($p<0.05$) and, in those livers in which tumor was present, the mean tumor volume was markedly reduced (2 mm^3 versus 884 mm^3, $p<0.05$). This effect was not seen when IL-2 was administered at a distant site nor in those mice receiving irradiated wild type controls. The effect was dose-dependent, with maximum inhibition seen at the highest IL-2 dose. Histologic examination revealed a marked infiltrate at the site within the livers with IL-2 secreting cells, comprised of both lymphocytes and Kupffer cells. In summary, these results demonstrate that local delivery of IL-2 inhibited tumor growth in this murine model of hepatic melanoma metastasis. This strategy has potential application for the development of immunotherapy of liver tumors.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

We claim:

1. A formulation for inhibiting the growth of tumors in a patient comprising:

a first cytokine in combination with an antigen in a pharmaceutical acceptable carrier for controlled and sustained release, and

a second cytokine in a pharmaceutical acceptable carrier for controlled and sustained release at the site of a tumor,

wherein the combination of cytokines is in an effective dosage to inhibit the growth of tumors in the patient.

2. The formulation of claim 1 wherein the first cytokine is granulocyte-macrophage colony-stimulating factor.

3. The formulation of claim 1 wherein the second cytokine is selected from the group consisting of interleukin-2 and interleukin-4.

4. The formulation of claim 1 further comprising separately administering tumor antigen with the cytokine.

5. The formulation of claim 4 wherein the tumor antigen is administered in the form of tumor cells rendered replication incompetent.

6. The formulation of claim 1 wherein cytokine is microencapsulated in a biocompatible controlled release polymeric matrix.

7. The formulation of claim 1 wherein the cytokine is administered in the form of cytokine secreting tumor cells rendered replication incompetent.

8. The formulation of claim 1 wherein the first cytokine is GM-CSF and the second cytokine is interleukin-2 released from transduced cells or microparticles.

9. A method for inhibiting tumor growth in a patient comprising:

administering to a patient in need of treatment a first cytokine in combination with an antigen, wherein the combination is administered in a controlled and sustained release formulation, and

administering to the patient a second cytokine in a controlled and sustained release formulation,

wherein the combination of the first cytokine and second cytokine inhibit tumor growth in the patient.

10. The method of claim 9 wherein the first cytokine is granulocyte-macrophage colony-stimulating factor.

11. The method of claim 9 wherein the second cytokine is selected from the group consisting of interleukin-2 and interleukin-4.

12. The method of claim 9 further comprising administering to the patient tumor antigen.

13. The method of claim 12 wherein the tumor antigen is in the form of cytokine secreting tumor cells rendered replication incompetent.

14. The method of claim 9 wherein cytokine is microencapsulated.

15. The method of claim 9 wherein the second cytokine is in the form of cytokine secreting tumor cells rendered replication incompetent.

16. The method of claim 14 wherein the second cytokine is microencapsulated in a controlled release formulation.

17. The method of claim 9 wherein the first cytokine is granulocyte-macrophage colony-stimulating factor and the second cytokine is interleukin-2.

18. The method of claim 9 wherein the patient has a brain tumor and the second cytokine is administered intracranially.

19. The method of claim 18 wherein the first cytokine is GM-CSF in combination with tumor antigen.

20. The method of claim 19 wherein the second cytokine is a microencapsulated interleukin.

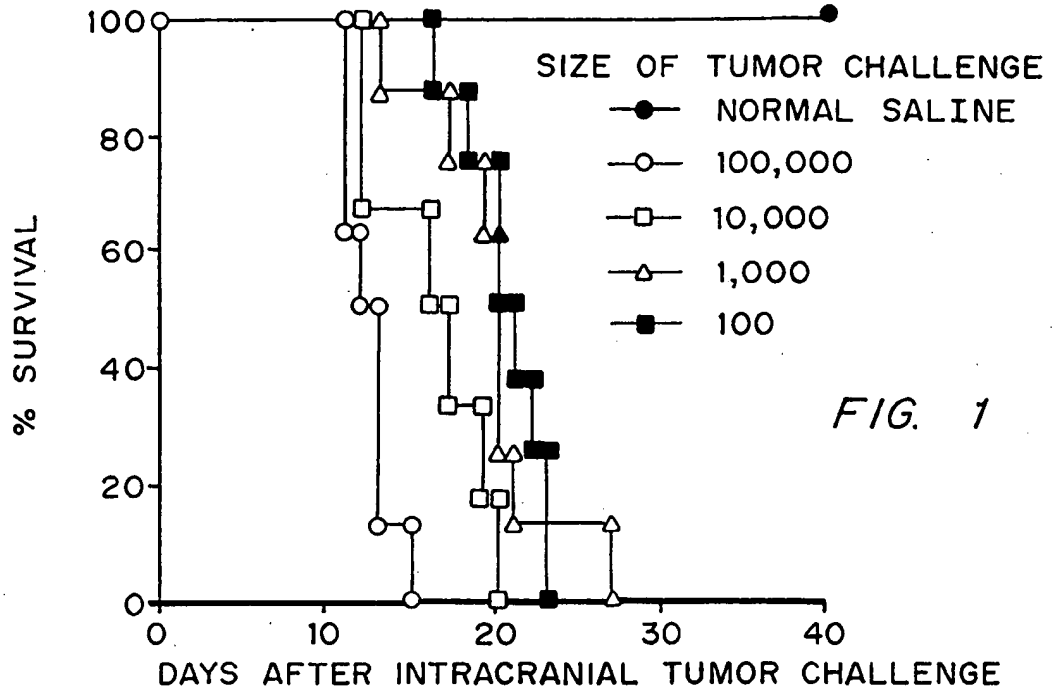

100
80
60
40
20
0
% SURVIVAL
0
10
20
30
40
DAYS AFTER INTRACRANIAL TUMOR CHALLENGE
SIZE OF TUMOR CHALLENGE
NORMAL SALINE
100,000
10,000
1,000
100

FIG. 1

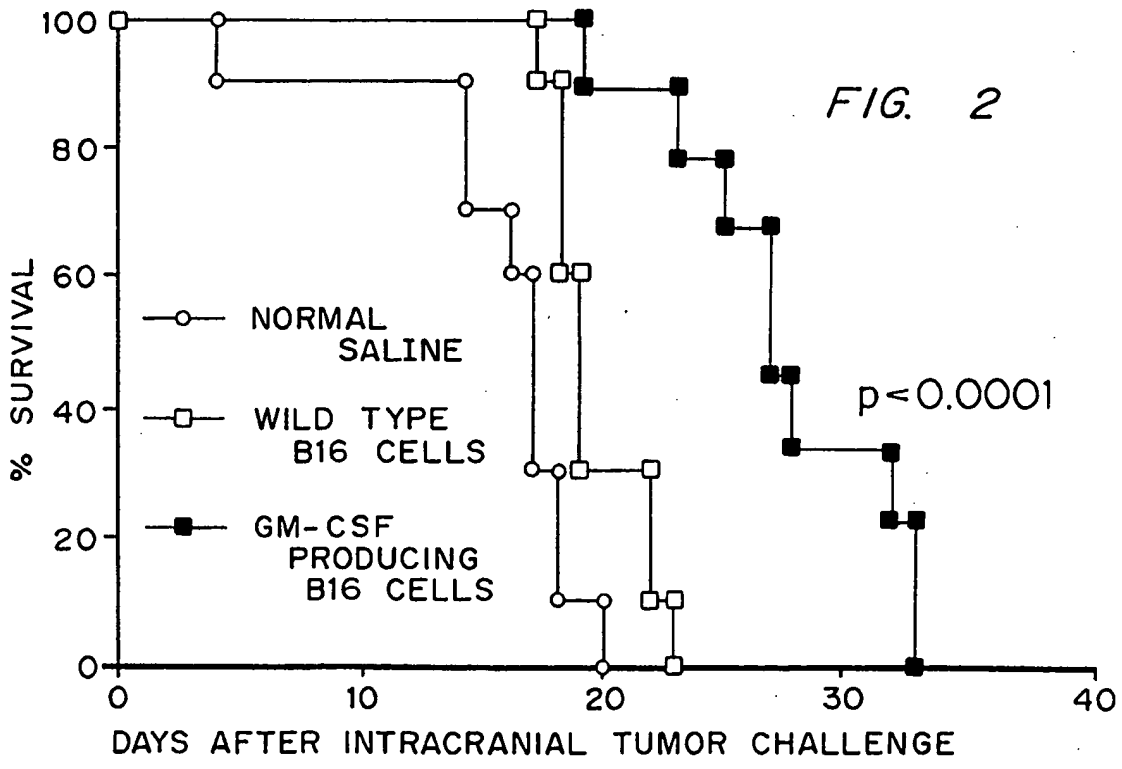

100
80
60
40
20
0
% SURVIVAL
0
10
20
30
40
DAYS AFTER INTRACRANIAL TUMOR CHALLENGE
NORMAL SALINE
WILD TYPE B16 CELLS
GM-CSF PRODUCING B16 CELLS
p<0.0001

FIG. 2

FIG. 3

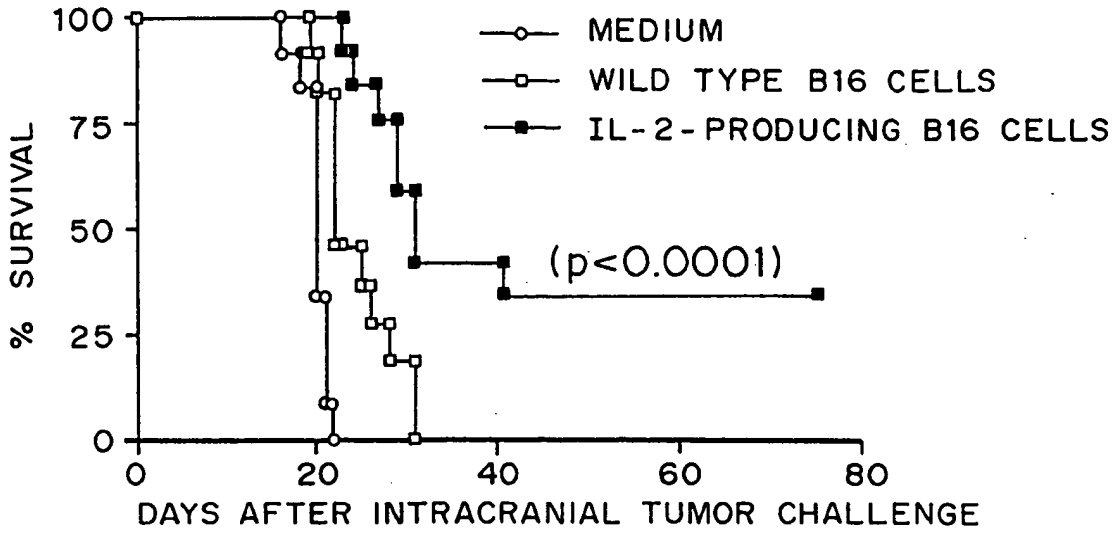
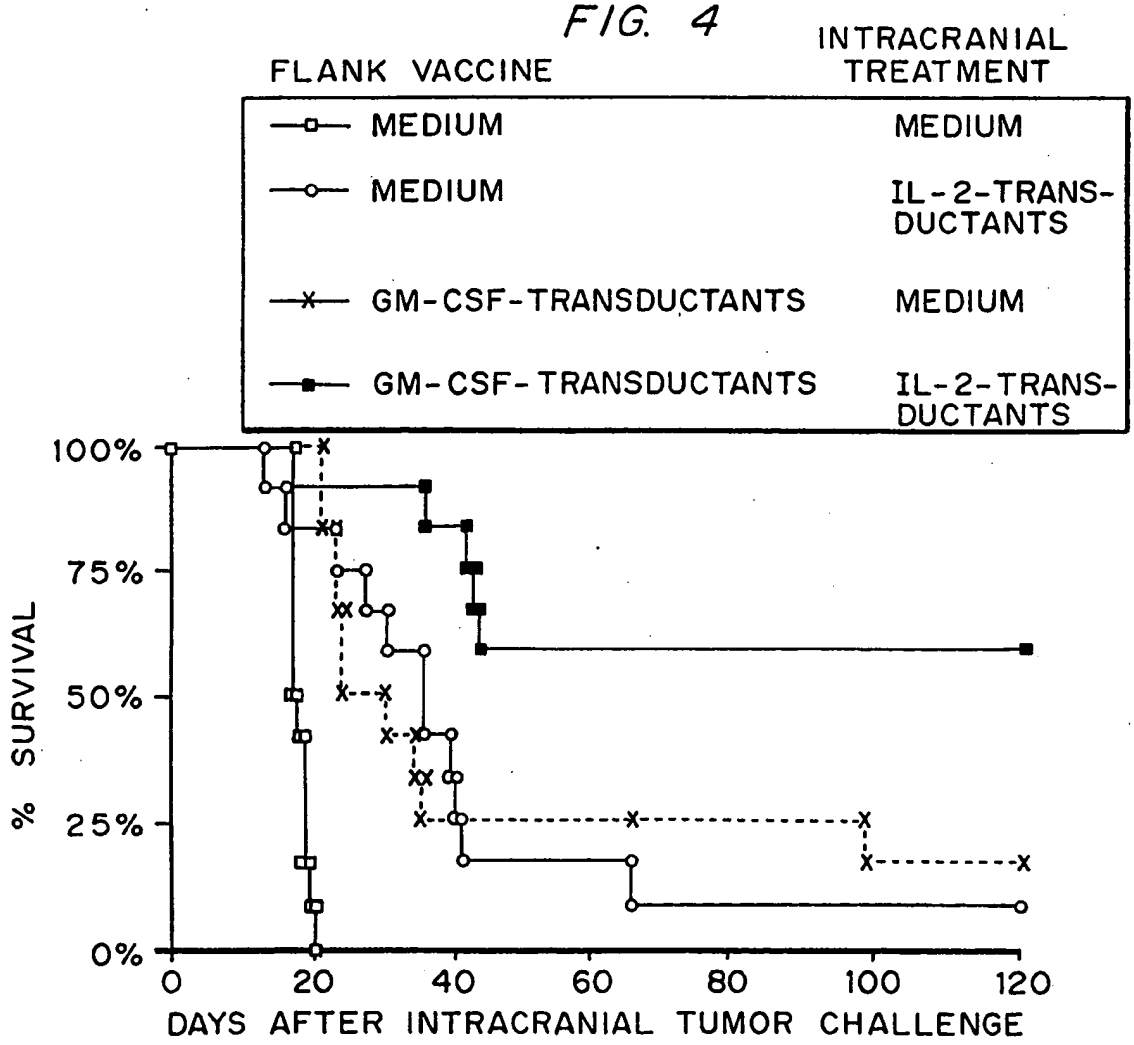

100
75
50
25
0
% SURVIVAL
MEDIUM
WILD TYPE B16 CELLS
IL-2-PRODUCING B16 CELLS
(p<0.0001)
0
20
40
60
80
DAYS AFTER INTRACRANIAL TUMOR CHALLENGE

FIG. 4


FLANK VACCINE
INTRACRANIAL TREATMENT
MEDIUM
MEDIUM
MEDIUM
IL-2-TRANS-DUCTANTS
GM-CSF-TRANSDUCTANTS
MEDIUM
GM-CSF-TRANSDUCTANTS
IL-2-TRANS-DUCTANTS
100%
75%
50%
25%
0%
% SURVIVAL
0
20
40
60
80
100
120
DAYS AFTER INTRACRANIAL TUMOR CHALLENGE

INTERNATIONAL SEARCH REPORT

Intern. nal Application No
PCT/US 97/18455

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/00 //A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PIPPIN ET AL: "LOCAL IL-4 DELIVERY ENHANCES IMMUNE REACTIVITY TO MURINE TUMORS: GENE THERAPY IN COMBINATION WITH IL2" CANCER GENE THERAPY, vol. 1, no. 1, 1994, pages 35-42, XP002057835 see the whole document ---	1-20
X	PAPPO ET AL: "ADMINISTRATION OF SYSTEMIC OR LOCAL INTERLEUKIN-2 ENHANCES THE ANTI-TUMOR EFFECTS OF INTERLEUKIN-12 GENE THERAPY" JOURNAL OF SURGICAL RESEARCH, vol. 58, 1995, pages 218-226, XP002057836 see the whole document --- -/--	1-20

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

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"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
5 March 1998	25. 03. 1998

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INTERNATIONAL SEARCH REPORT

Inten ɔnal Application No
PCT/US 97/18455

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VAGLIANI ET AL: "INTERLEUKIN 12 POTENTIATES THE CURATIVE EFFECT OF A VACCINE BASED ON INTERLEUKIN 2-TRANSDUCED TUMOR CELLS" CANCER RESEARCH, vol. 56, 1 February 1996, pages 467-470, XP002057837 see the whole document ---	1-20
X	HOLLINGSWORTH ET AL: "THE EFFECT OF COMBINED EXPRESSSION OF INTERLEUKIN 2 AND INTERLEUKIN 4 ON THE TUMORIGENICITY AND TREATMENT OF B16F10 MELANOMA" BRITISH JOURNAL OF CANCER, vol. 74, July 1996, pages 6-15, XP002057838 see the whole document ---	1-20
X	BELLDEGRUN ET AL: "HUMAN RENAL CARCINOMA LINE TRANSFECTED WITH INTERLEUKIN-2 AND/OR INTERFERON ALPHA GENE(S): IMPLICATIONS FOR LIVE CANCER VACCINES" JOURNAL OF THE NATIONAL CANCER INSTITUTE, vol. 85, 1993, pages 207-216, XP002057839 see the whole document ---	1-20
X	DRANOFF ET AL: "VACCINATION WITH IRRADIATED TUMOR CELLS ENGINEERED TO SECRETE MURINE GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR STIMULATES POTENT, SPECIFIC, AND LONG-LASTING ANTI-TUMOR IMMUNITY" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES,USA, vol. 90, 1993, pages 3539-3543, XP002057840 cited in the application see the whole document ---	1-20
A	GOLUMBEK ET AL: "CONTROLLED RELEASE, BIODEGRADABLE CYTOKINE DEPOTS: A NEW APPROACH IN CANCER VACCINE DESIGN" CANCER RESEARCH, vol. 53, 1993, pages 5841-5844, XP002057841 see page 5841 see abstract --- -/--	

1

INTERNATIONAL SEARCH REPORT

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PCT/US 97/18455

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	THOMPSON ET AL: "SYSTEMIC AND LOCAL PARACRINE CYTOKINE THERAPIES USING TRANSDUCED TUMOR CELLS ARE SYNERGISTIC IN TREATING INTRACRANIAL TUMORS" JOURNAL OF IMMUNOTHERAPY, vol. 19, no. 6, November 1996, pages 405-413, XP002057842 see the whole document -----	1-20

1

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 97/18455

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Remark : Although claims 9-20 are directed to a method of treatment of the human/animal body , the search has been carried out and based on the alleged effects of the compound/composition.

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(81) Designated States: AL, AU, BA, BB, BG, BR, CA, CN, CU, CZ, EE, GE, HU, IL, IS, JP, KP, KR, LC, LK, LR, LT, LV, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, SL, TR, TT, UA, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

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(54) Title: CYTOKINE ENHANCED IMMUNOTHERAPY FOR BRAIN TUMORS

(57) Abstract

A therapy for treatment of patients with cancer utilizing the combination of a cytokine in a pharmaceutically acceptable carrier for systemic administration and a cytokine in a pharmaceutically acceptable carrier for local administration is described. In the most preferred embodiment, brain tumors are treated with a cytokine such as GM-CSF administered systemically, most preferably in combination with tumor antigen such as replication incompetent tumor cells, and a cytokine such as IL-2 or IL-4 administered locally, most preferably in a vehicle providing release over a period of time, such as transduced cells, again most preferably replication incompetent tumor cells, or incorporated into microparticulate vehicles such as polymeric microspheres.

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CYTOKINE ENHANCED IMMUNOTHERAPY FOR BRAIN TUMORS

Field of the Invention

This invention is in the field of treating cancer by administering a combination of systemic and local immunotherapy using cytokines.

Rights of the Federal Government

The United States government has certain rights in this invention by virtue of a grant by the National Cooperative Drug Discovery Group UO1-CA52857 of the National Cancer Institute of the National Institutes of Health, Bethesda, Maryland.

Background of the Invention

One-third of all individuals in the United States alone will develop cancer. Although the five year survival rate has risen dramatically to nearly fifty percent as a result of progress in early diagnosis and therapy, cancer still remains second only to cardiac disease as a cause of death in the United States. Twenty percent of Americans die from cancer, half due to lung, breast, and colon-rectal cancer.

Designing effective treatments for patients with cancer has represented a major challenge. The current regimen of surgical resection, external beam radiation therapy, and/or systemic chemotherapy has been partially successful in some kinds of malignancies, but has not produced satisfactory results in others. In some malignancies, such as brain malignancies, this regimen produces a median survival of less than one year. For example, 90% of resected malignant gliomas recur within two centimeters of the original tumor site within one year.

Though effective in some kinds of cancers, the use of systemic chemotherapy has had minor success in the treatment of cancer of the colon-rectum, esophagus, liver, pancreas, and kidney. A major problem with systemic chemotherapy for the treatment of these types of cancer is that the systemic doses required to achieve control of tumor growth frequently result in unacceptable systemic toxicity. Efforts to improve delivery of

chemotherapeutic agents to the tumor site have resulted in advances in organ-directed chemotherapy, as by continuous systemic infusion, for example. However, continuous infusions of anticancer drugs generally have not shown a clear benefit over pulse or short-term infusions. Implantable elastomer access ports with self-sealing silicone diaphragms have also been tried for continuous infusion, but extravasation remains a problem. Portable infusion pumps are now available as delivery devices and are being evaluated for efficacy. (See Harrison's Principles of Internal Medicine, pp. 431-446, Braunwald, E., et al., ed., McGraw-Hill Book Co. (1987), for a general review). Controlled release biocompatible polymers have been used successfully for local drug delivery and have been utilized for contraception, insulin therapy, glaucoma treatment, asthma therapy, prevention of dental related disorders, and certain types of cancer chemotherapy. (Langer, R., and Wise, D., eds, Medical Applications of Controlled Release, Vol. I and II, Boca Raton, CRC Press (1986)).

In the brain, the design and development of effective anti-tumor agents for treatment of patients with malignant neoplasms of the central nervous system have been influenced by two major factors: 1) the blood-brain barrier provides an anatomic obstruction, limiting access of drugs to these tumors; and 2) the drugs given at high systemic levels are generally cytotoxic. Efforts to improve drug delivery to the tumor bed in the brain have included transient osmotic disruption of the blood brain barrier, cerebrospinal fluid perfusion, and direct infusion into a brain tumor using catheters. Each technique has had significant limitations. Disruption of the blood brain barrier increased the uptake of hydrophilic substances into normal brain, but did not significantly increase substance transfer into the tumor. Only small fractions of agents administered into the cerebrospinal fluid actually penetrated into the brain parenchyma. Drugs that have been used to treat tumors by infusion have been inadequate, did not diffuse an adequate distance from the site of infusion, or could not be maintained at a sufficient concentration to allow a sustained diffusion gradient. The use of catheters has been complicated by high rates of infection, obstruction, and malfunction

due to clogging. See Tomita, T., "Interstitial chemotherapy for brain tumors: review", *J. Neuro-Oncology,* 10:57-74 (1991).

Recent advances in the understanding of the immune system and advances in defining T cell antigens on tumor cells have shown promising results in treating tumors with immunotherapy. Several of these new approaches are aimed at augmenting weak host immune responses to tumor antigens and include the use of antibodies, cellular immunotherapy, and cytokines. The use of antibodies is difficult because it requires that the specificity of the antibody be such that it does not significantly bind to non-tumor cells. There are few truly tumor-specific antigens to select when an antibody-based immunotherapy approach is designed. Cellular immunotherapy involves the transfer of cultured immune cells that have anti-tumor reactivity into a tumor-bearing host. Adoptive therapy with autologous lymphokine activated killer (LAK) cells has yielded impressive results but only in the presence of cytokines or chemotherapeutic drugs. The use of cytokines administered directly to cells to enhance immune responses has also been shown to be successful. See Abbas, et al., "Immunity to Tumors", page 372, Cellular and Molecular Immunology, 2nd Ed., W.B. Saunders Company (1994). However, the effective administration of cytokines suffers from the same obstacles discussed above in reference to administration of chemotherapeutic agents in that high doses of systemic administration can be toxic and providing continuous release by local administration can be problematic.

It is therefore an object of the present invention to provide a composition and method of use thereof which provides for an enhanced immune response against tumors, especially brain tumors, improves therapeutic efficacy and diminishes potential toxicity.

It is a further object of the present invention to provide a composition and method of use for the treatment of tumors, especially brain tumors, which establishes long term memory capable of generating potent anti-tumor responses against multiple subsequent tumor challenges.

Summary of the Invention

A therapy for treatment of patients with cancer utilizing the combination of a cytokine in a pharmaceutical acceptable carrier for systemic administration and a cytokine in a pharmaceutical acceptable carrier for local administration is described. In the most preferred embodiment, brain tumors are treated with a cytokine such as granulocyte macrophage-colony stimulating factor (GM-CSF) administered systemically, most preferably in combination with tumor antigen such as replication incompetent tumor cells, and a cytokine such as interleukin-2 (IL-2) or IL-4 administered locally, most preferably in a vehicle providing release over a period of time, such as transduced cells, again most preferably replication incompetent tumor cells, or incorporated into microparticulate vehicles such as polymeric microspheres.

The examples demonstrate that systemic administration (vaccination) with GM-CSF transduced tumor cells or microencapsulated GM-CSF protects against growth of intracranial melanoma. The examples also demonstrate that local intracranial delivery of IL-2 transduced tumor cells or microencapsulated IL-2 generates immediate anti-tumor responses within the central nervous system as well as long term memory capable of generating potent anti-tumor responses against multiple subsequent tumor challenges, including challenges outside the central nervous system. The examples further demonstrate that combination immunotherapy using systemic vaccination with GM-CSF transductants and local intracranial administration of IL-2 transductants produces an anti-tumor effect that is significantly enhanced as compared to treatment with either treatment alone.

Brief Description of the Drawings

Figure 1 is a graph showing percent survival over time in days of experimental animal models of intracranial melanoma having received stereotactic intracranial injections of wild type B16-F10 melanoma cells in doses of 100,000 (circles), 10,000 (squares), 1,000 (triangles), and 100 (solid squares), and saline with no cells (solid circles).

Figure 2 is a graph showing percent survival over time in days of mice after systemic vaccination with a single subcutaneous injection of 10^6 irradiated B16-F10 cells engineered by gene transfer to secrete GM-CSF (solid squares), medium (circles) or 10^6 irradiated B16-F10 wild type non-cytokine producing cells (squares), challenged 14 days later with an injection of 10^2 non-irradiated wild type B16-F10 melanoma cells in the brain, ($p < 0.001$).

Figure 3 is a graph showing percent survival over time in days of mice treated with a single intracranial injection of 10^5 irradiated B16-F10 cells engineered by gene transfer to secrete IL-2 (solid squares), medium (circles) or 10^5 irradiated wild type B16-F10 non-cytokine producing cells (squares), challenged at the same time by stereotactic intracranial co-injections of 10^2 non-irradiated wild type B16-F10 cells, ($p < 0.001$).

Figure 4 is a graph showing percent survival over time in days of animals vaccinated with a subcutaneous injection of 10^6 irradiated GM-CSF producing B16-F10 melanoma cells followed two weeks later with 5×10^4 irradiated IL-2-secreting B16-F10 cells administered intracranially and simultaneously challenged with intracranial co-injections of 10^2 non-irradiated wild type B16-F10 cells (solid squares). Control animals received GM-CSF vaccine alone followed by intracranial tumor challenge with wild type B16-F10 cells (x marks), or vaccination with medium alone followed by intracranial IL-2 therapy and tumor challenge (circles). Additional controls received vaccination with medium followed by intracranial therapy with medium and intracranial tumor challenge (squares).

Detailed Description of the Invention

A therapy for treatment of tumors has been developed which relies on the combination of an initial systemic "priming" of the immune system, most preferably through the combination of administration of a cytokine such as GM-CSF and tumor antigen such as replication incompetent tumor cells along with local release at the tumor site (or site of resection following tumor removal) of a cytokine such as IL-2 which enhances the immune response against the tumor cells. Local release can be obtained using any of several

means, but a preferred method is using microparticles to release cytokine over a period of at least days or transduced cells, most preferably replication incompetent tumor cells which are transduced with the gene encoding the cytokine to be released. The latter is shown to release for at least five days after implantation. Microparticles can be designed to release for between hours and weeks or even months, as required.

The examples show the *in vivo* treatment of brain tumors using GM-CSF administered systemically, and IL-2 and IL-4 administered intracranially. However, a variety of cytokines can be used. Similar results are expected for treatment of other tumor types.

I. Therapeutic Compositions

a. Cytokines

i. Granulocyte-macrophage colony stimulating factor. Granulocyte-macrophage colony stimulating factor (GM-CSF) is a 22 kD glycoprotein made by activated T cells and by activated mononuclear phagocytes, vascular endothelial cells, and fibroblasts. GM-CSF has been shown to prime systemic immune responses *via* stimulation of bone marrow derived antigen presenting cells. See Inaba, K., et al., *J. Exp. Med.*, 175:1157-67 (1992); Inaba, K., et al., *J. Exp. Med.*, 176:1693-702 (1992); Steinman, R., *Annual Rev. Immunology*, 9:271-81 (1991). *In vivo* studies with GM-CSF-transduced tumor cells reveal that local release of this cytokine results in the generation of CD4+ and CD8+ tumor-specific T lymphocytes and systemic protection from tumor challenge. Dranoff, G., et al., *Proc. Natl. Acad. Sci. U.S.A.*, 90:3539-43 (1993).

ii. Interleukin-2. Interleukin-2 (IL-2) is produced by CD4+ T cells, and in lesser quantities by CD8+ T cells. Secreted IL-2 is a 14 to 17 kD glycoprotein encoded by a single gene on chromosome 4 in humans. IL-2 acts on the same cells that produce it, i.e., it functions as an autocrine growth factor. IL-2 also acts on other T lymphocytes, including both CD4+ and CD8+ cells. IL-2 induces a local inflammatory response leading to activation of both helper and cytotoxic subsets of T cells. IL-2 also stimulates the growth of natural killer cells and enhances their cytolytic

function. *In vivo* studies with IL-2-secreting tumor cells demonstrate that powerful local and systemic antitumor immune responses are generated leading to the destruction of the transduced tumors in the flank. Fearon, E. R., et al., *Cell*, 60:397-403 (1990); Gansbacher, B., et al., *J. Exp. Med.*, 172:1217-24 (1990).

iii. Tumor necrosis factor. Tumor necrosis factor (TNF) was originally identified as a mediator of tumor necrosis present in the serum of animals exposed to bacterial lipopolysaccharide (LPS) such as endotoxin. The major endogenous source of TNF is the LPS-activated mononuclear phagocyte, although antigen-stimulated T cells, activated natural killer cells, and activated mast cells can also secrete this protein. In the mononuclear phagocyte, TNF is initially synthesized as a nonglycosylated transmembrane protein of approximately 25 kD. TNF has potent anti-tumor effects *in vitro*, although clinical trials of TNF in advanced cancer patients have been discontinued due to toxicity. TNF-α has a diverse range of biological properties including inducing expression of a number of cytokines such as interleukin-6, interleukin-8, GM-CSF, and granulocye-colony stimulating factor, as well as causing hemorrhagic necrosis in established tumors. TNF has been reported to generate tumor suppression after tumor cell-targeted TNF-α gene transfer. Blankenstein, T., et al., *J. Exp. Med.*, 173:1047-52 (1991).

iv. Interleukin-4. Interleukin-4 (IL-4) is a helper T cell-derived cytokine of approximately 20 kD which stimulates the proliferation of mouse B cells in the presence of anti-Ig antibody (an analog of antigen) and causes enlargement of resting B cells as well as increased expression of class II MHC molecules. The principal endogenous source of IL-4 is from CD4+ T lymphocytes. Activated mast cells and basophils, as well as some CD8+ T cells, are also capable of producing IL-4. IL-4 delivered intracranially displays antitumor activity analogous to observations following administration peripherally of IL-4 transduced tumors. Golumbek, P.T., et al., *Science*, 254:713-6 (1991); Yu, J.S., et al., *Cancer Res.*, 53:3125-8 (1993).

v. Gamma interferon. Gamma interferon (IFN-γ) is a homodimeric glycoprotein containing approximately 21 to 24 kD subunits. IFN-γ is produced by some CD4+ helper T cells and nearly all CD8+ T cells. Transcription is directly initiated as a consequence of antigen activation and is enhanced by IL-2 and interleukin-12. IFN-γ is also produced by natural killer cells. IFN-γ acts as a potent activator of mononuclear phagocytes, acts directly on T and B lymphocytes to promote their differentiation and acts to stimulate the cytolytic activity of natural killer cells. IFN-γ transduced non-immunogenic sarcoma has been reported to elicit CD8+ T cells against wild type tumor cells. Restifo, N., et al., *J. Exp. Med.*, 175:1423-28 (1992).

vi. Interleukin-3. Interleukin-3 (IL-3), also known as multilineage colony-stimulating factor, is a 20 to 26 kD product of CD4+ T cells that acts on the most immature marrow progenitors and promotes the expansion of cells that differentiate into all known mature cell types. IL-3 has been reported to enhance development of tumor reactive cytotoxic T cells by a CD4-dependent mechanism. Pulaski, B. A., et al., *Cancer Res.*, 53:2112-57 (1993).

vii. Interleukin-6. Interleukin-6 (IL-6) is a cytokine of approximately 26 kD that is synthesized by mononuclear phagocytes, vascular endothelial cells, fibroblasts, and other cells in response to IL-1 and, to a lesser extent, TNF. It is also made by some activated T cells. IL-6 transfected into Lewis lung carcinoma tumor cells has been reported to suppress the malignant phenotype and to confer immunotherapeutic competence against parental metastatic cells. Porgador, A., *Cancer Res.*, 52:3679-87 (1992).

viii. Interleukin-7. Interleukin-7 (IL-7) is a cytokine secreted by marrow stromal cells that acts on hematopoietic progenitors committed to the B lymphocyte lineage. IL-7 has been reported to induce CD42+ T cell dependent tumor rejection. Hock, H., et al., *J. Exp. Med.*, 174:1291-99 (1991).

ix. Granulocyte-colony stimulating factor. Granulocyte-colony stimulating factor (G-CSF) is made by the same cells that make GM-CSF. The secreted polypeptide is approximately 19 kD. G-CSF gene transfer has been reported to suppress tumorgenicity of murine adenocarcinoma. Colombo, M., et al., Cancer Res., 52:4853-57 (1991).

x. Other cytokines. Other cytokines are known in the art to have an anti-tumor effect and can be used in the pharmaceutical compositions described herein. Moreover, since cytokines are known to have an effect on other cytokines, one can administer the cytokine which elicits one of the cytokines described above, or directly administer one of the cytokines which is elicited. Additional cytokines are known to those skilled in the art and are described in Abbas, et al., "Cytokines", chapter 12, pp. 239-61, Cellular and Molecular Immunology, 2nd Ed., W.B. Saunders Company (1994).

b. Combinations with other biologically active compounds

The cytokines can also be administered in combination with other cytokines, antibodies, cultured immune cells that have anti-tumor reactivity including LAK cells, or chemotherapeutic agents, including radiation therapy. The active compounds can be incorporated into the same vehicle as the cytokines for administration, or administered in a separate vehicle.

i. Chemotherapeutic agents.

The cytokines can be used alone, or in combination with other chemotherapeutic agents. Examples of chemotherapeutic agents include cytotoxic agents such as paclitaxel, camptothecin, ternozolamide, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), adriamycin, platinum drugs such as cisplatin, differentiating agents such as butyrate derivatives, transforming growth factor such as factor-alpha-*Pseudomonas* exotoxin fusion protein, and antibodies to tumor antigens, especially glioma antigens, such as monoclonal antibody 81C6. These agents can be incorporated into polymeric matrices for delivery along with the cytokines. See, for example, Domb, et al., *Polym. Prepr.*, 32(2):219-220 (1991), reported incorporating the water soluble chemotherapeutic agents carboplatin, an analog of cisplatin, and 4-

hydroperoxycyclophosphamide into a biodegradable polymer matrix for treating tumors, with promising results in.

ii. Other pharmaceutically active compounds.

In variations of these embodiments, it may be desirable to include other pharmaceutically active compounds, such as steroidal antiinflammatories which are used to reduce swelling, antibiotics, antivirals, or anti-angiogenic compounds. For example, dexamethasone, a synthetic corticosteroid used systemically to control cerebral edema, has been incorporated into a non-biodegradable polymer matrix and tested in rat brain *in vitro* and *in vivo* for efficacy in reversing cerebral edema. Other compounds which can be included are preservatives, antioxidants, and fillers, coatings or bulking agents which may also be utilized to alter polymeric release rates.

c. Cytokine Formulations

The cytokines can be administered in a pharmaceutically acceptable carrier such as saline, phosphate buffered saline, cells transduced with a gene encoding the cytokine, microparticles, or other conventional vehicles.

i. Polymeric formulations

The cytokines can be encapsulated into a biocompatible polymeric matrix, most preferably biodegradable, for use in the treatment of solid tumors. The cytokine is preferably released by diffusion and/or degradation over a therapeutically effective time, usually eight hours to five years, preferably one week to one year. As used herein, microencapsulated includes incorporated onto or into or on microspheres, microparticles, or microcapsules. Microcapsules is used interchangeably with microspheres and microparticles, although it is understood that those skilled in the art of encapsulation will recognize the differences in formulation methods, release characteristics, and composition between these various modalities. The microspheres can be directly implanted or delivered in a physiologically compatible solution such as saline.

Biocompatible polymers can be categorized as biodegradable and non-biodegradable. Biodegradable polymers degrade *in vivo* as a function of

chemical composition, method of manufacture, and implant structure. Synthetic and natural polymers can be used although synthetic polymers may be preferred due to more uniform and reproducible degradation and other physical properties. Examples of synthetic polymers include polyanhydrides, polyhydroxyacids such as polylactic acid, polyglycolic acid and copolymers thereof, polyesters, polyamides, polyorthoesters, and some polyphosphazenes. Examples of naturally occurring polymers include proteins and polysaccharides such as collagen, hyaluronic acid, albumin and gelatin. The ideal polymer must also be strong, yet flexible enough so that it does not crumble or fragment during use.

Cytokines and optionally, other drugs or additives, can be encapsulated within, throughout, and/or on the surface of the implant. The cytokine is released by diffusion, degradation of the polymer, or a combination thereof. There are two general classes of biodegradable polymers: those degrading by bulk erosion and those degrading by surface erosion. The latter polymers are preferred where more linear release is required. The time of release can be manipulated by altering chemical composition; for example, by increasing the amount of an aromatic monomer such as p-carboxyphenoxy propane (CPP) which is copolymerized with a monomer such as sebacic acid (SA). A particularly preferred polymer is CPP-SA (20:80). Use of polyanhydrides in controlled delivery devices has been reported by Leong, et al., *J. Med. Biomed. Mater. Res.*, 19:941 (1985); *J. Med. Biomed. Mater. Res.*, 20:51 (1986); and Rosen, et al., *Biomaterials*, 4:131 (1983). U.S. Patents that describe the use of polyanhydrides for controlled delivery of substances include U.S. Patent 4,857,311 to Domb and Langer, U.S. Patent 4,888,176 to Langer, et al., and U.S. Patent 4,789,724 to Domb and Langer. Other polymers such as polylactic acid, polyglycolic acid, and copolymers thereof have been commercially available as suture materials for a number of years and can be readily formed into devices for drug delivery.

Non-biodegradable polymers remain intact *in vivo* for extended periods of time (years). Agents loaded into the non-biodegradable polymer

matrix are released by diffusion through the polymer's micropore lattice in a sustained and predictable fashion, which can be tailored to provide a rapid or a slower release rate by altering the percent cytokine loading, porosity of the matrix, and implant structure. Ethylene-vinyl acetate copolymer (EVAc) is an example of a nonbiodegradable polymer that has been used as a local delivery system for proteins and other macromolecules, as reported by Langer, R., and Folkman, J., *Nature (London)*, 263:797-799 (1976). Others include polyurethanes, polyacrylonitriles, and some polyphosphazenes.

In the preferred embodiment, only polymer and cytokines to be released are incorporated into the delivery device, although other biocompatible, preferably biodegradable or metabolizable, materials can be included for processing purposes as well as additional therapeutic agents.

Buffers, acids and bases are used to adjust the pH of the composition. Agents to increase the diffusion distance of agents released from the implanted polymer can also be included.

Fillers are water soluble or insoluble materials incorporated into the formulation to add bulk. Types of fillers include sugars, starches and celluloses. The amount of filler in the formulation will typically be in the range of between about 1 and about 90% by weight.

Spheronization enhancers facilitate the production of spherical implants. Substances such as zein, microcrystalline cellulose or microcrystalline cellulose co-processed with sodium carboxymethyl cellulose confer plasticity to the formulation as well as implant strength and integrity. During spheronization, extrudates that are rigid, but not plastic, result in the formation of dumbbell shaped implants and/or a high proportion of fines. Extrudates that are plastic, but not rigid, tend to agglomerate and form excessively large implants. A balance between rigidity and plasticity must be maintained. The percent of spheronization enhancer in a formulation depends on the other excipient characteristics and is typically in the range of 10 to 90% (w/w).

Disintegrants are substances which, in the presence of liquid, promote the disruption of the implants. The function of the disintegrant is to

counteract or neutralize the effect of any binding materials used in the formulation. The mechanism of disintegration involves, in large part, moisture absorption and swelling by an insoluble material. Examples of disintegrants include croscarmellose sodium and crospovidone which are typically incorporated into implants in the range of 1 to 20% of total implant weight. In many cases, soluble fillers such as sugars (mannitol and lactose) can also be added to facilitate disintegration of the implants.

Surfactants may be necessary in implant formulations to enhance wettability of poorly soluble or hydrophobic materials. Surfactants such as polysorbates or sodium lauryl sulfate are, if necessary, used in low concentrations, generally less than 5%.

Binders are adhesive materials that are incorporated in implant formulations to bind powders and maintain implant integrity. Binders may be added as dry powder or as solution. Sugars and natural and synthetic polymers may act as binders. Materials added specifically as binders are generally included in the range of about 0.5 to 15% w/w of the implant formulation. Certain materials, such as microcrystalline cellulose, also used as a spheronization enhancer, also have additional binding properties.

Various coatings can be applied to modify the properties of the implants. Three types of coatings are seal, gloss and enteric. The seal coat prevents excess moisture uptake by the implants during the application of aqueous based enteric coatings. The gloss coat improves the handling of the finished product. Water-soluble materials such as hydroxypropyl cellulose can be used to seal coat and gloss coat implants. The seal coat and gloss coat are generally sprayed onto the implants until an increase in weight between about 0.5% and about 5%, preferably about 1% for seal coat and about 3% for a gloss coat, has been obtained.

Enteric coatings consist of polymers which are insoluble in the low pH (less than 3.0) of the stomach, but are soluble in the elevated pH (greater than 4.0) of the small intestine. Polymers such as Eudragit®, RohmTech, Inc., Malden, MA, and Aquateric®, FMC Corp., Philadelphia, PA, can be used and are layered as thin membranes onto the implants from aqueous

solution or suspension. The enteric coat is generally sprayed to a weight increase of about one to about 30%, preferably about 10 to about 15%, and can contain coating adjuvants such as plasticizers, surfactants, separating agents that reduce the tackiness of the implants during coating, and coating permeability adjusters. Other types of coatings having various dissolution or erosion properties can be used to further modify implant behavior. Such coatings are readily known to one of ordinary skill in the art.

Controlled release devices are typically prepared in one of several ways. For example, the polymer can be melted, mixed with the substance to be delivered, and then solidified by cooling. Such melt fabrication processes require polymers having a melting point that is below the temperature at which the substance to be delivered and polymer degrade or become reactive. Alternatively, the device can be prepared by solvent casting, where the polymer is dissolved in a solvent, and the substance to be delivered dissolved or dispersed in the polymer solution. The solvent is then evaporated, leaving the substance in the polymeric matrix. Solvent casting requires that the polymer be soluble in organic solvents and that the agents to be encapsulated be soluble or dispersible in the solvent. Similar devices can be made by phase separation or emulsification or even spray drying techniques. In still other methods, a powder of the polymer is mixed with the cytokine and then compressed to form an implant.

Methods of producing implants also include granulation, extrusion, and spheronization. A dry powder blend is produced including the desired excipients and microspheres. The dry powder is granulated with water or other non-solvents for microspheres such as oils and passed through an extruder forming "strings" or "fibers" of wet massed material as it passes through the extruder screen. The extrudate strings are placed in a spheronizer which forms spherical particles by breakage of the strings and repeated contact between the particles, the spheronizer walls and the rotating spheronizer base plate. The implants are dried and screened to remove aggregates and fines. These methods can be used to make micro-implants

(microparticles, microspheres, and microcapsules encapsulating cytokines to be released), slabs or sheets, films, tubes, and other structures.

ii. Cytokine secreting Genetically engineered cells

Cytokine secreting cells can be genetically engineered by methods known in the art to deliver cytokines systemically or locally. Cells can either be transformed or transduced, with bacterial or viral DNA vectors, respectively. In a preferred embodiment the cells are tumor cells. To prevent replication and preserve cytokine production, the transduced cells can be irradiated prior to *in vivo* injection. The advantage of the tumor cells is that the patient is exposed to antigen in combination with cytokine stimulating the immune response to the cytokine. Examples of the preparation of tumor cells engineered to secrete IL-4 are described in Golumbek, P. T., et al., *Science*, 254:713-6 (1991) and Yu, J. S., et al., *Cancer Res.* 53:3125-8 (1993). IL-2 gene transfer into tumor cells is described in Gansbacher, B., et al., *J. Exp. Med.*, 172:1217-24 (1990). Tumor cells engineered to secrete GM-CSF are described in Dranoff, G., et al., *Proc. Natl. Acad. Sci. U.S.A.*, 90:3539-43 (1993). TNF gene transfer is described in Blankenstein, T., et al., *J. Exp. Med.*, 73:1047-52 (1991). IL-6 transfection into lung carcinoma tumor cells is described in Progador, A, et al., *Cancer Res.*, 52:3679-87 (1992). γIFN cDNA transduced into non-immunogenic sarcoma is described in Restifo, N., *J. Exp. Med.*, 175:1423-28 (1992). G-CSF gene transfer is described in Colombo, M., et al., Cancer Res., 52:4853-57 (1991). Other cytokines including IL-3 and IL-7 can be secreted from cells by applying the methods used for the cytokines described above or by the general procedures for genetically modifying nonimmunogenic murine fibrosarcoma described in Karp, S. E., et al., *J. Immunol.*, 150:896-908 (1993) and Jaffee, E. et al., *J. Immunotherapy*, in press (1995). The publications cited above describe methods of genetically engineering cells to secrete cytokines. Additionally, cancer vaccines expressing two or more cytokines from engineered vectors containing genes encoding two different cytokines or by sequential recombinant retrovirus-mediated genetic transductions can be prepared. Suitable pharmaceutical vehicles are known to those skilled in the art.

Inducers can also be used to produce cells which secrete cytokines. Inducers may be used alone in some cases or in combination with transforming agents, and include tumor necrosis factor (TNF), endotoxin, and other agents known to those skilled in the art. In general, the cultured cells are exposed to an amount effective to activate the cells, as determined by cytokine expression, immunoglobulin secretion, and/or other indicators such as proliferation or alteration of cell surface properties or markers. In some cases, cells are initially exposed to a small amount to "prime" the cells, then to a subsequent dose to elicit greater activation of the cells.

Cells can be administered in medium or washed and administered in saline. Alternatively, cells can be encapsulated in a polymeric matrix and administered.

II. Administration to Patients

The cytokines described herein or their functionally equivalent derivatives can be administered alone or in combination with, either before, simultaneously, or subsequent to, treatment using other chemotherapeutic or radiation therapy or surgery. A preferred embodiment is systemic administration of a first cytokine such as GM-CSF, most preferably in combination with tumor antigen in the form of replication incompetent tumor cells, followed by local administration during surgery or by injection of either a biocompatible polymeric matrix loaded with the selected cytokine or cytokine secreting cells, using dosages determined as described herein. Local administration can be at a site adjacent to the tumor to be removed, or in the tumor, or in the place where the tumor was removed. The dosages for functionally equivalent derivatives can be extrapolated from the *in vivo* data. An effective amount is an amount sufficient to induce an anti-tumor effect as measured either by a longer survival time or decrease in tumor size.

Local administration can also be accomplished using an infusion pump, for example, of the type used for delivering insulin or chemotherapeutic agents to specific organs or tumors.

In the preferred method of administration, the polymeric implants or cytokine secreting cells are implanted at the site of a tumor, two weeks after

systemic administration of the polymeric implants or cytokine secreting cells, for example, by subcutaneous injection at a distant site. If biodegradable polymers are used, preferably they are less than about 100 to 200 microns in diameter and injected by means of a catheter or syringe; it is not necessary to remove the implant following release of the immunotherapeutic agent.

The cytokines can also be combined with other therapeutic modalities, including radiotherapy, chemotherapeutic agents administered systemically or locally, and other immunotherapy.

The cytokines are preferably administered to brain cancer patients. However, the cytokine combination therapy can also be applied to all cancer patients including those having breast cancer, lung cancer, and colon cancer. The treatment will provide an immediate response at the site of the local administration as well as provide long term memory protection against cancer at the site of local administration and at a site distant from the site of local administration. The immunotherapy described herein is therefore useful in preventing or treating metastases.

III. Examples.

The present invention will be further understood by reference to the following non-limiting examples.

Example 1: Systemic vaccination with GM-CSF-transduced tumor cells

Tumor cell lines and animals. B16-F10 melanoma cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum and penicillin/streptomycin. Animals used for all experiments were 6 to 12 week old C57BL/6 female mice obtained from Harlan (Indianapolis, IN). B16-F10 melanoma cells were transduced with murine GM-CSF gene by using the replication-defective MFG retroviral vector as previously described in Dranoff, G., et al., *Proc. Natl. Acad. Sci. U.S.A.*, 90:3539-43 (1993). The amount of cytokine produced by the transformed tumor cells was quantified routinely by a standard ELISA technique (Endogen, Cambridge, MA). Cultured tumor monolayers were harvested with trypsin and resuspended in DMEM before injection. Tumor cells were exposed to 5000 rads from a ^{137}cesium source (Gammacell Model

#62 irradiator, Nordin International, Inc., Kanata, Ontario, Canada) discharging 1378 rads per minute, immediately before injection in order to render them replication incompetent.

Technique for intracranial cell injections. Mice were anesthetized with an intraperitoneal injection of 0.1 ml of a stock solution containing ketamine hydrochloride 25 mg/ml, xylazine 2.5 mg/ml, and 14.25% ethyl alcohol diluted 1:3 in 0.9% NaCl solution. For stereotactic intracranial injections of tumor cells, the surgical site was shaved and prepared with 70% ethyl alcohol and prepodyne solution. After a midline incision, a 1 mm burr hole center 2 mm posterior to the coronal suture and 2 mm lateral to the sagittal suture was made. Animals were then placed in a stereotactic frame and cells were delivered by a 26 gauge needle to a depth of 3 mm over a period of 3 minutes. The total volume of injected cells was 10 μl. The needle was removed, the site irrigated with sterile 0.9% NaCl solution, and the skin was sutured closed with 4.0 vicryl.

Development of an intracranial B16-F10 melanoma model in C57BL/6 mice. To assess the intracranial growth characteristics of B16-F10 melanoma, animals were divided into five groups of at least eight animals and received stereotactic intracranial injections of either 10^2, 10^3, 10^4, or 10^5 wild type B16-F10 melanoma cells into the left parietal region by the method described above. Control animals received similar intracranial injections of 0.9% NaCl solution. Animals were assessed daily for survival. For histologic analysis, brains were removed at the time of death and fixed in 10% formalin for at least 5 days, sectioned, embedded in paraffin, and stained with hematoxylin and eosin.

All animals receiving the highest dose of B16-F10 (10^5 cells) died within 15 days of treatment, with a median survival of 12 days. The results are shown in Figure 1. At doses of 10^3 and 10^4 cells the median survival was 20 and 16 days, respectively. Intracranial does of 10^2 cells were uniformly fatal (median survival 20 days, range 16 to 21 days). These kinetics emphasize the highly tumorigenic, poorly immunogenic character of

B16-F10. All control animals (treated with stereotactic injections of 0.9% NaCl) were alive at 120 days.

Histologic analysis of the brains revealed that all animals treated with intracranial wild type B16-F10 developed large, solid tumor masses at the injection in the parietal region. Metastatic deposits of tumor were occasionally observed in the brain parenchyma distant from the injection site, especially in those animals receiving large tumor inoculations. Tumor deposits were also observed in the subarachnoid space surrounding the spinal cord, suggesting that cell migration occurred along cerebrospinal fluid pathways. No tumor was apparent outside the craniospinal axis. This new model has proven to be highly reliable and reproducible. With intracranial doses of 10^2 tumor cells, animals died consistently over a 3 to 4 day period within 20 days of injection.

Vaccination studies. Cytokine-secreting B16-F10 melanoma cells were evaluated for their vaccination properties and ability to protect against intracranial challenge with wild type B16-F10 melanoma. For these studies, animals were treated with a single subcutaneous flank injection of 10^6 irradiated GM-CSF secreting B16-F10 cells using a tuberculin syringe with a 27 gauge needle. The total amount of GM-CSF secreted by the transduced cells *in vitro* prior to injection was between 50 and 60 ng per 10^6 cells per day. To prevent replication and preserve cytokine production, the transduced cells were irradiated prior to *in vivo* injection. Previous studies had shown that B16-F10 melanoma cells exposed to 5000 rads are rendered replication incompetent, but maintain their metabolic viability and continue to secrete cytokine *in vivo* for up to 5 days, Jaffee, E. et al., "Use of murine models of cytokine-secreting tumor vaccines to study feasibility and toxicity issues critical to designing clinical trials", *J. Immunotherapy*, in press (1995). The experiments were conducted with two control groups: one group received flank injections of 10^6 irradiated wild type (non-cytokine-producing) B16 cells and the other was treated with a subcutaneous injection of either cell growth medium or 0.9% NaCl. There were at least 10 animals per group for all experiments. After 2 weeks all animals were challenged with stereotactic

intracranial injections of 10^2 non-irradiated wild type B16-F10 melanoma cells by the method outlined above. Animals were assessed for survival.

Statistical analysis. For all efficacy studies, survival was plotted using a Kaplan-Meier survival analysis and statistical significance was determined by the Kruskal-Wallis nonparametric analysis of variance as described in Zar, J., eds., Biostatistical Analysis, Englewood Cliffs, New Jersey, Prentice-Hall, Inc., pp. 140-50 (1984). Percentages of long term survivors were analyzed by comparison of two proportions.

Results

Figure 2 shows the results of the systemic vaccination studies performed on animals receiving single subcutaneous injections of 10^6 irradiated B16-F10 cells engineered by gene transfer to secrete GM-CSF. GM-CSF secreting cells administered in this manner were found to protect against intracranial challenge with the wild type tumor. Median survival for animals treated with the GM-CSF vaccine and then challenged 2 weeks later with wild type B16 in the brain was 27 days, compared to 19 and 17 days for control animals treated with a vaccine of irradiated, wild type cells (which did not secrete GM-CSF) or medium, respectively ($p<0.0001$). These results have been replicated 4 times, each with similar and statistically significant results.

Example 2: Systemic vaccination with microencapsulated GM-CSF

Thirty-two C57BL/6 mice were divided into four groups of eight and given flank vaccinations with either: 1) saline, 2) 2500 ng bolus GM-CSF and 1 x 10^6 irradiated B16 melanoma cells, 3) 2500 ng GM-CSF microspheres and 1 x 10^6 irradiated melanoma cells, or 4) 1 x 10^6 irradiated melanoma cells transduced to produce GM-CSF. Fourteen days later all animals received a challenge of B16 melanoma in the left frontal lobe.

Results

The median survival of the GM-CSF microsphere group and the transduced GM-CSF secreting group was 27.5 days each. These survival periods are significantly prolonged compared to the control animals receiving saline which survived 16.5 days, ($p<0.001$). The GM-CSF bolus group

survived 15.5 days, therefore having no effect on survival. No GM-CSF treated animal showed any sign of toxicity from the vaccination.

This study demonstrates that a significant anti-tumor immune response is generated using a systemic vaccination with GM-CSF microspheres. This response is comparable to that generated by GM-CSF transduced tumor cells and represents a practical method for delivering GM-CSF in treatments employing GM-CSF immunotherapy.

Example 3: Local intracranial delivery of IL-2-transduced tumor cells

Animals received intracranial stereotactic co-injections of either 10^3, 10^4, 10^5, or 10^6 irradiated cytokine-producing B16-F10 melanoma cells, which produce 80 ng of IL-2 per 10^6 cells every twenty-four hours as determined by ELISA. Control animals received co-injections of irradiated non-IL-2 producing wild type B16-F10 at the equivalent doses of 10^3, 10^4, 10^5, or 10^6 cells. Simultaneously, all animals were challenged with stereotactic intracranial injections of 10^2 live non-irradiated B16-F10 melanoma cells. For these experiments, there were at least 10 animals per group.

Results

IL-2 transduced cells were found to be highly effective in treating intracranial tumor when they were delivered directly to the brain. Initial studies showed that the IL-2 effect was dose-dependent. The results are shown in Figure 3. Doses of 0.08 ng per day of IL-2 (10^3 IL-2 producing cells) showed no enhanced survival compared to controls, whereas a dose of 0.8 ng per day (10^4 IL-2 producing cells) showed a trend toward prolonged survival that did not reach statistical significance. When tested at a dose of 8.0 ng per day (10^5 IL-2 producing cells), a significant prolongation in survival was seen. While untreated control animals had died with intracranial tumor by day 22 (median survival, 20 days), median survival for the IL-2 group was 31 days with 33% of the mice living more than 80 days, ($p < 0.001$). Interestingly, several animals that died in the IL-2 treatment group were found to have no tumor in the brain; rather, tumor was observed surrounding the spinal cord suggesting that the protective effect of IL-2 was

localized to the site of cytokine delivery. At higher doses of IL-2 (80.0 ng per day), signs of IL-2 toxicity were observed including ataxia and death. These animals died before controls (median survival, 12 days). These studies have been replicated 7 times with similar doses of IL-2 transductants. In each experiment, there was a significant prolongation of survival in animals treated with intracranial IL-2 transductants as compared to controls.

On histologic examination at the time of death, inflammation was notably absent in the brain parenchyma of the treated animals. Lymphocytes were observed, however, in the cerebrospinal fluid spaces of the IL-2 treated animals.

Example 4: Intracranial delivery of microencapsulated interleukin-2 produces long term memory and protects against re-challenges

IL-2 was incorporated into biodegradable sustained release microspheres having an average diameter of 4 μm. These microspheres were stereotactically injected into the brains of mice to deliver 100 ng of IL-2 over a 7 day period. Safety testing showed no evidence of clinical or histologic toxicity from this dose over a 120 day period. To evaluate efficacy as a primary treatment, a lethal challenge of B16 melanoma was stereotactically co-injected into the left parietal lobe of 25 mice with either saline, empty microspheres, or the IL-2 containing microspheres. In a similar experiment, 9L glioma cells were co-injected into the left parietal lobe of rats.

To evaluate the potential brain vaccine effect of this therapy, a group of non tumor-bearing mice were co-injected intracranially with IL-2 microspheres and melanoma cells which had been irradiated to prevent replication. Thirty days later these animals and a group of control mice having no previous treatment were challenged intracranially with the lethal dose of live melanoma with no additional IL-2 therapy.

Results

Seventy-five percent of the IL-2 treated mice (6 of 8) were alive with no sign of tumor 90 days after initial treatment while median survival of untreated controls was 18 days. No animal survived more than 21 days. The empty microspheres had no effect on survival. In a similar mouse

melanoma experiment, no impact on survival from either a bolus injection of IL-2 to the tumor site or systemic IL-2 administration was observed. In the glioma experiments, microsphere delivery of IL-2 prolonged median survival in rats to 55 days compared to 25 days for the controls.

Previous IL-2 vaccination extended median survival by 45 percent, with 2 to 5 animals alive at 160 days after tumor challenge with all controls dead by 23 days.

These results indicate that brain interstitial immunotherapy with IL-2 delivered by microspheres is an effective and safe treatment against brain metastases and inhibits tumor recurrence. Additional efficacy results and release kinetics of IL-2 from microspheres are reported in Sills, A. K., Jr., et al., *Proceed. Intern. Symp. Control. Rel. Biomater.*, 23 (July, 1996), and Kalyanasundaram, S., et al., *Proceed. Intern. Symp. Control. Rel. Biomater.*, 23 (July, 1996), respectively.

Example 5: Intracranial delivery of IL-2 transduced cells produces long term memory and protects against re-challenges

A group of 11 C57BL/6 mice were initially treated with intracranial IL-2 and co-injected with a dose of B16 melanoma that was uniformly fatal in control animals. Unlike controls, all 11 animals who received intracranial IL-2 survived the initial tumor challenge in the brain and were rechallenged at the same site on day 70. Six of the 11 were rechallenged with wild-type melanoma cells. The other five received injections of media alone. Twelve additional mice, previously untreated, were similarly challenged with B16 melanoma in the brain.

Results

The melanoma challenge was fatal to all previously untreated mice, the median survival was 17 days. By contrast, the mice initially treated with IL-2 and rechallenged with tumor on day 70 had a significantly prolonged survival where the median survival was 36 days, with 3 of 6 surviving 70 days ($p=0.01$ versus positive control, Mann-Whitney Test). As expected, mice rechallenged with media all lived an additional 70 days.

These results demonstrate long-term memory in an immune response directed against intracranial melanoma useful in preventing metastatic tumor recurrence after resection or inhibiting growth of synchronous metastases by delivery of IL-2 transduced cells.

Example 6: Local intracranial delivery of IL-2 transduced cells protects against challenges outside the central nervous system

Twenty-four C57B16 mice were injected intracranially with irradiated, IL-2 secreting B16-F10 melanoma cells and 12 C57B16 mice were injected with medium, as controls. All animals simultaneously received a co-injection of wild type B16-F10 cells which were uniformly fatal to untreated mice. Nine of the twenty-four IL-2 treated animals survived 75 days. No controls survived more than 20 days. At day 75, the 9 surviving animals and 8 new control animals were challenged in the flank with 10,000 wild type B16-F10 melanoma cells.

Results

Only 1 of 9 long-term survivors developed a flank tumor; in contrast, 6 of 8 control mice developed a flank tumor, ($p=.024$). The 8 (of 9) long-term survivors who rejected the first flank challenge, along with 8 new control animals, were challenged a second time in the flank with a larger bolus of 50,000 wild type B16-F10 melanoma cells, 126 days after the first challenge. Seventeen days later, 8 of 8 control mice had developed tumors whereas only 2 of 8 of the long term survivors had a flank tumor ($p=0.01$).

This study demonstrates that localized IL-2 immunotherapy delivered intracranially not only generates an immediate anti-tumor response within the central nervous system, but also establishes long-term memory capable of generating potent anti-tumor responses against multiple subsequent tumor challenges outside the central nervous system.

Example 7: Local intracranial delivery of IL-4-transduced tumor cells

Poorly immunogenic B16-F10 melanoma cells were transfected with the IL-4 gene to deliver IL-4 at the site of a tumor in the central nervous system. The transfected B16-F10 cells were irradiated to prevent replication and injected intracranially in eight C57BL/6 mice along with non-irradiated,

non-transfected B16-F10 (wild type) melanoma cells. The expression of IL-4 receptors on B16-F10 melanoma cells was examined by means of fluorescence activated cell sorting (FACS).

Results

While animals treated with IL-4 had a median survival of 31 days, control animals that received only wild type cells had a median survival of 18 days (range 15 to 20 days; $p=0.002$).

FACS analysis of B16-F10 cells did not reveal the presence of the IL-4 receptor, suggesting that IL-4 does not interact with its receptor on tumor cells to exert the cytotoxic effect. The results obtained in these experiments indicate that high levels of IL-4 expressed locally stimulate a strong immunologic antitumor response which leads to significant prolongation of survival in mice with central nervous system tumors.

Example 8: Combination therapy: Systemic vaccination with GM-CSF transductants and local intracranial administration of IL-2 transductants

For combination experiments, all animals received a subcutaneous flank vaccination and were challenged after two weeks with the standard intracranial dose of 10^2 wild type melanoma cells co-injected with the specific intracranial treatment. The flank vaccination was with a single subcutaneous injection of 10^6 irradiated GM-CSF-producing cells and the intracranial treatment two weeks later was with 5×10^4 irradiated IL-2 producing cells injected into the brain. Given that a dose of 8.0 ng per day of IL-2 (10^5 cells), yielded a 33 percent long-term survivor rate and approached doses at which toxicity was observed, a lower dose of 4.0 ng per day of IL-2 (5×10^4 cells) was selected. Control animals received either the subcutaneous GM-CSF vaccine and intracranial treatment with medium, or subcutaneous vaccination with medium and intracranial treatment with 5×10^4 IL-2 producing cells. Additional controls received vaccinations of medium alone followed by intracranial treatment with medium along with an intracranial tumor challenge.

Results

When animals were treated with the GM-CSF-secreting cell vaccine in combination with intracranial IL-2-producing cells, a greatly enhanced response was achieved. The results are shown in Figure 4. Median survival was not yet reached at 100 days, with 58% of the mice still alive after combination therapy. This represents a significant advantage in survival over flank vaccination with GM-CSF-producing cells alone (median survival, 27 days, $p=0.018$), over local intracranial administration of IL-2-producing cells alone (median survival, 35 days, $p=0.01$), and over control (median survival, 17.5 days, $p<0.001$). The rate of 58% of long term survivors, those surviving more than 100 days, in the combination group exceeds the percentage in the GM-CSF flank vaccine group (16%, $p=0.08$), the intracranial IL-2 transductant group (8.3%, $p=0.03$), or the sum of the two, providing a greatly enhanced response. Both the mice receiving the GM-CSF vaccine alone and those treated with intracranial IL-2-secreting cells alone showed prolongation of survival compared to controls (IL-2, $p=0.0006$; GM-CSF, $p<0.001$), confirming the efficacy of single cytokine therapy in this model.

These results indicate that the GM-CSF vaccine generates a cohort of CD4+ and CD8+ T-cells specific for a tumor antigen, which activity of the cells is enhanced by the subsequent local IL-2 therapy at the tumor site. The results correspond with the theory that while the blood-brain barrier poses an obstacle to delivery of chemotherapeutic agents to the brain, it functions as an avenue of entry into the central nervous system for immunologically active cells. Wekerly, H., et al., *TINS,* 27:1-7 (1986).

The degree of inflammatory infiltrate in the brain parenchyma of animals treated with these cytokines was small, regardless of the mode of delivery. These findings are in contrast to studies of cytokine enhanced antitumor immune responses done in flank models, where a marked local inflammatory infiltrate was apparent. These results indicate that intrinsic immune response cells within the brain such as microglia may also be involved in the protective immune response.

Example 9: Systemic vaccination with GM-CSF transductants and local intracranial administration of IL-2 transductants produces long term memory protection against re-challenges

Genetically engineered non-replicating B16-F10 melanoma cells were used for systemic delivery of GM-CSF or intracranial delivery of IL-2. Forty-nine C57B16 mice were vaccinated in the flank with either GM-CSF producing B16-F10 cells or medium followed by intracranial delivery of IL-2 or medium 2 weeks later. Every animal received an intracranial co-injection of wild-type melanoma at a dose uniformly fatal to untreated animals. Initial survivors and a new control group were rechallenged with a second intracranial dose of wild-type B16-F10 melanoma 134 days after their first challenge.

Results

Animals receiving both flank GM-CSF and intracranial IL-2 had the longest survival, with median survival not yet reached at 130 days (7 of 13 alive). This is a significant improvement over either flank GM-CSF alone (median survival 27 days, $p=0.018$), intracranial IL-2 alone (32 days, $p=0.01$) or control (17.5 days, $p<0.001$). GM-CSF alone and IL-2 alone were significantly more effective than the controls, ($p<0.006$).

Seven animals who received combination therapy with GM-CSF and IL-2 survived more than 130 days. Five of the 7 mice rechallenged were alive 28 days later. All 8 controls died by day 21 ($p=0.018$). Furthermore, the antitumor effect persisted for greater than 4 months. These results demonstrate that use of cytokines can lead to immunologic memory in the brain.

Example 10: Interstitial delivery of IL-2 to the brain

Methods

An alternative approach to the control of metastatic brain tumors by using local immunotherapy with interstitial delivery of interleukin-2 (IL-2) as both a primary treatment for establishing tumors and a vaccine against subsequent recurrence is described. IL-2 was incorporated into biodegradable sustained release microspheres (average diameter 4 μm) which were stereotactically injected into the brains of mice to deliver 100 ng IL-2 over a

7 day period. Safety testing showed no evidence of clinical or histologic toxicity from this dose over a 120 day period. To evaluate efficacy as a primary treatment, a lethal challenge of B16 melanoma was stereotactically co-injected into the left parietal lobe of 25 mice with either: saline (controls), empty (no IL-2) microspheres, or the IL-2 containing microspheres.

Results

75% of the IL-2 treated animals (6/8) were alive with no sign of tumor 90 days after initial treatment while median survival of untreated controls was 18 days with no animal surviving more than 21 days. The empty microspheres had no effect on survival. In a similar mouse melanoma test, no impact was seen on survival from either bolus injection of IL-2 to the tumor site or systemic IL-2 administration, suggesting that the sustained local delivery of IL-2 in the brain provided by the microspheres is important for the continued stimulation of immune cells attracted to the tumor bed.

Example 11: Intracranial injections of IL-2 into the brain

Methods

To evaluate the potential brain vaccine effect of this therapy, a group of non tumor-bearing mice were co-injected intracranially with IL-2 microspheres and melanoma cells (see example 10) which had been irradiated to prevent replication. 30 days later these animals and a group of control mice (no previous treatment) were challenged intracranially with the lethal dose of live melanoma with no additional IL-2 therapy.

Results

Previous IL-2 vaccination extended median survival by 45% and generated 2/5 animals alive at 160 days after tumor challenge with all controls dead by 23 days. These results suggest that brain interstitial immunotherapy with IL-2 delivered by microspheres is an effective and safe treatment against brain metastases and may prevent tumor recurrence.

Example 12: Combination therapy with locally delivered IL-2 and chemotherapeutic agents

Methods

A combination therapy consisting of immunotherapy, using non-replicating genetically engineered tumor cells that produce IL-2, and local delivery of chemotherapy was tested to determine if the component therapies acted synergistically against an intracranial tumor challenge in a murine brain tumor model. B16-F10 melanoma cells were transduced with murine IL-2 using a replication-defective MFG retroviral vector. Chemotherapeutic agents, BCNU and carboplatin, were incorporated into controlled-release polymers. C57/B16 mice received intracranial challenge of wild-type tumor with and without non-replicating IL-2 producing tumor cells. On day 5, animals received polymer implants containing 4.0% BCNU, 1% carboplatin, or placebo.

Results

Animals receiving IL-2 and 1% carboplatin (n=10, median survival greater than 100 days, $p<0.01$) had significantly improved survival over animals receiving IL-2 (n=10, median survival 38 days) or 1% carboplatin alone (n=, median survival 23 days). Animals receiving IL-2 and 4.0% BCNU (n=10, median survival 44 days) had improved survival over animals receiving 4.0% BCNU (n=10, median survival 24 days) or IL-2 alone (n=10, median survival 38 days). The median survival for the control group (n=10) was 19 days. This example demonstrates that combination intracranial immunotherapy with IL-2 and local delivery of chemotherapy improves survival over either antitumor therapy alone. This effect was obtained even when utilizing low doses of chemotherapy (1% carboplatin and 4.0% BCNU).

Example 13: IL-2 inhibited tumor growth in murine model of hepatic melanoma metastasis

Methods

Useful immunotherapeutic strategies must demonstrate success in tumors within the liver, an organ with unique immunologic properties and a common site of metastases in humans. The anti-tumor effects of local or

paracrine IL-2 delivery was evaluated using the non-immunogenic B16-F10 melanoma implanted into the liver of C57B1/6 mice. Irradiated autologous tumor cells transfected with the IL-2 gene was used as the source of IL-2. Varying numbers of these cells (5×10^5 to 2×10^6) were admixed with 1×10^4 wild type B16-F10 melanoma cells and injected intrahepatically, and compared to cells alone, cells coinjected with non-transfected cells, or with IL-2 cells injected at a distant site (flank) (n=7/group). Presence or absence of tumor within the liver and tumor volumes were measured on day 21.

Results

Inhibition of tumor growth was seen in mice receiving locally delivered IL-2 at all doses. The number of mice without any tumor was significantly less in those receiving the highest IL-2 dose ($p<0.05$) and, in those livers in which tumor was present, the mean tumor volume was markedly reduced (2 mm^3 versus 884 mm^3, $p<0.05$). This effect was not seen when IL-2 was administered at a distant site nor in those mice receiving irradiated wild type controls. The effect was dose-dependent, with maximum inhibition seen at the highest IL-2 dose. Histologic examination revealed a marked infiltrate at the site within the livers with IL-2 secreting cells, comprised of both lymphocytes and Kupffer cells. In summary, these results demonstrate that local delivery of IL-2 inhibited tumor growth in this murine model of hepatic melanoma metastasis. This strategy has potential application for the development of immunotherapy of liver tumors.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

We claim:

1. A formulation for inhibiting the growth of tumors in a patient comprising:

a first cytokine in combination with an antigen in a pharmaceutical acceptable carrier for controlled and sustained release, and

a second cytokine in a pharmaceutical acceptable carrier for controlled and sustained release at the site of a tumor,

wherein the combination of cytokines is in an effective dosage to inhibit the growth of tumors in the patient.

2. The formulation of claim 1 wherein the first cytokine is granulocyte-macrophage colony-stimulating factor.

3. The formulation of claim 1 wherein the second cytokine is selected from the group consisting of interleukin-2 and interleukin-4.

4. The formulation of claim 1 further comprising separately administering tumor antigen with the cytokine.

5. The formulation of claim 4 wherein the tumor antigen is administered in the form of tumor cells rendered replication incompetent.

6. The formulation of claim 1 wherein cytokine is microencapsulated in a biocompatible controlled release polymeric matrix.

7. The formulation of claim 1 wherein the cytokine is administered in the form of cytokine secreting tumor cells rendered replication incompetent.

8. The formulation of claim 1 wherein the first cytokine is GM-CSF and the second cytokine is interleukin-2 released from transduced cells or microparticles.

9. A method for inhibiting tumor growth in a patient comprising:

administering to a patient in need of treatment a first cytokine in combination with an antigen, wherein the combination is administered in a controlled and sustained release formulation, and

administering to the patient a second cytokine in a controlled and sustained release formulation,

wherein the combination of the first cytokine and second cytokine inhibit tumor growth in the patient.

10. The method of claim 9 wherein the first cytokine is granulocyte-macrophage colony-stimulating factor.

11. The method of claim 9 wherein the second cytokine is selected from the group consisting of interleukin-2 and interleukin-4.

12. The method of claim 9 further comprising administering to the patient tumor antigen.

13. The method of claim 12 wherein the tumor antigen is in the form of cytokine secreting tumor cells rendered replication incompetent.

14. The method of claim 9 wherein cytokine is microencapsulated.

15. The method of claim 9 wherein the second cytokine is in the form of cytokine secreting tumor cells rendered replication incompetent.

16. The method of claim 14 wherein the second cytokine is microencapsulated in a controlled release formulation.

17. The method of claim 9 wherein the first cytokine is granulocyte-macrophage colony-stimulating factor and the second cytokine is interleukin-2.

18. The method of claim 9 wherein the patient has a brain tumor and the second cytokine is administered intracranially.

19. The method of claim 18 wherein the first cytokine is GM-CSF in combination with tumor antigen.

20. The method of claim 19 wherein the second cytokine is a microencapsulated interleukin.

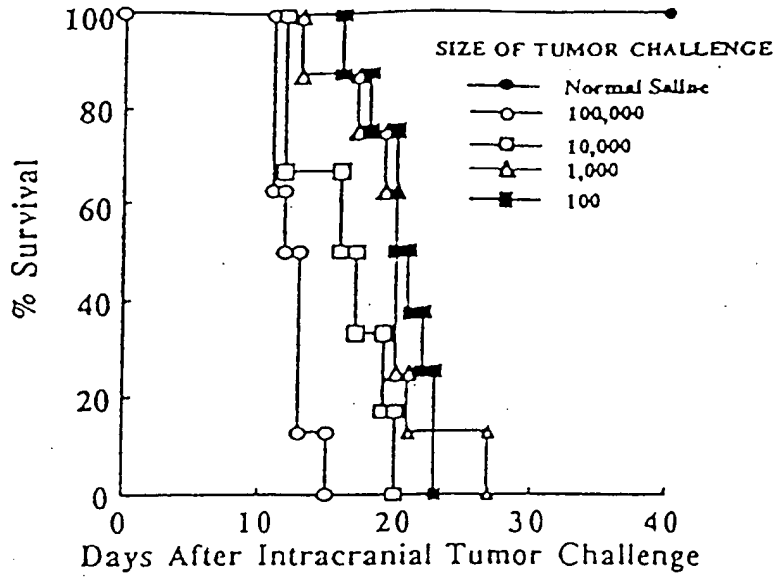
SIZE OF TUMOR CHALLENGE
Normal Saline
100,000
10,000
1,000
100
% Survival
100
80
60
40
20
0
0
10
20
30
40
Days After Intracranial Tumor Challenge

FIG. 1

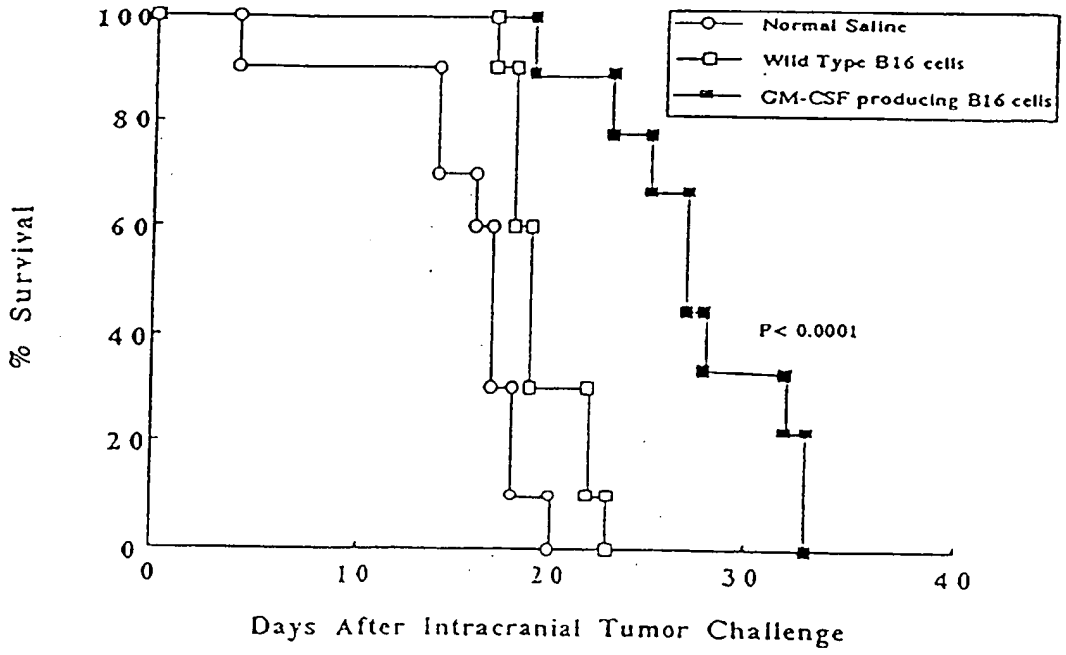
Normal Saline
Wild Type B16 cells
GM-CSF producing B16 cells
P< 0.0001
% Survival
100
80
60
40
20
0
0
10
20
30
40
Days After Intracranial Tumor Challenge

FIG. 2

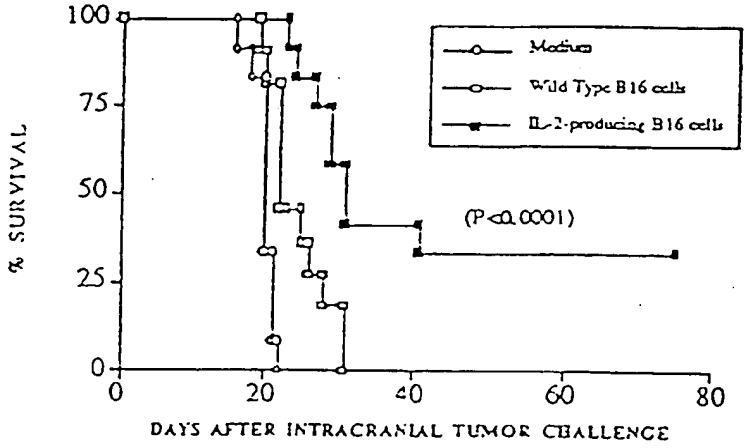
100
75
50
25
0
% SURVIVAL
0
20
40
60
80
DAYS AFTER INTRACRANIAL TUMOR CHALLENGE
(P<0.0001)
Medium
Wild Type B16 cells
IL-2-producing B16 cells

FIG. 3

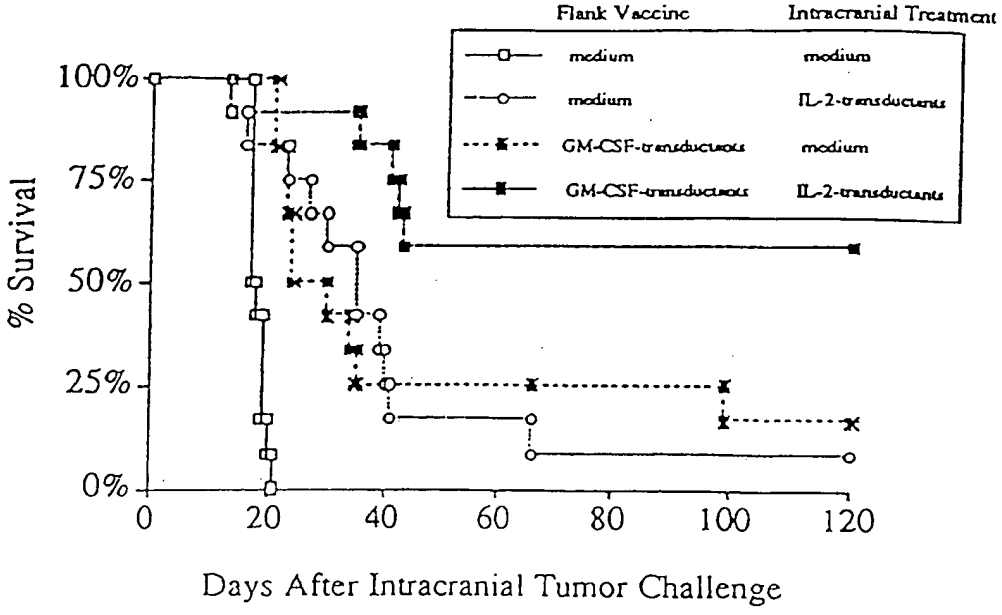
Flank Vaccine
Intracranial Treatment
medium
medium
medium
IL-2-transductants
GM-CSF-transductants
medium
GM-CSF-transductants
IL-2-transductants
100%
75%
50%
25%
0%
% Survival
0
20
40
60
80
100
120
Days After Intracranial Tumor Challenge

FIG. 4

INTERNATIONAL SEARCH REPORT

Interr. ...nal Application No
PCT/US 97/18455

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/00 //A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PIPPIN ET AL: "LOCAL IL-4 DELIVERY ENHANCES IMMUNE REACTIVITY TO MURINE TUMORS: GENE THERAPY IN COMBINATION WITH IL2" CANCER GENE THERAPY, vol. 1, no. 1, 1994, pages 35-42, XP002057835 see the whole document ---	1-20
X	PAPPO ET AL: "ADMINISTRATION OF SYSTEMIC OR LOCAL INTERLEUKIN-2 ENHANCES THE ANTI-TUMOR EFFECTS OF INTERLEUKIN-12 GENE THERAPY" JOURNAL OF SURGICAL RESEARCH, vol. 58, 1995, pages 218-226, XP002057836 see the whole document ---	1-20
	-/--	

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
5 March 1998	25. 03. 1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Sitch, W

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INTERNATIONAL SEARCH REPORT

Inten ɔnal Application No
PCT/US 97/18455

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VAGLIANI ET AL: "INTERLEUKIN 12 POTENTIATES THE CURATIVE EFFECT OF A VACCINE BASED ON INTERLEUKIN 2-TRANSDUCED TUMOR CELLS" CANCER RESEARCH, vol. 56, 1 February 1996, pages 467-470, XP002057837 see the whole document ---	1-20
X	HOLLINGSWORTH ET AL: "THE EFFECT OF COMBINED EXPRESSSION OF INTERLEUKIN 2 AND INTERLEUKIN 4 ON THE TUMORIGENICITY AND TREATMENT OF B16F10 MELANOMA" BRITISH JOURNAL OF CANCER, vol. 74, July 1996, pages 6-15, XP002057838 see the whole document ---	1-20
X	BELLDEGRUN ET AL: "HUMAN RENAL CARCINOMA LINE TRANSFECTED WITH INTERLEUKIN-2 AND/OR INTERFERON ALPHA GENE(S): IMPLICATIONS FOR LIVE CANCER VACCINES" JOURNAL OF THE NATIONAL CANCER INSTITUTE, vol. 85, 1993, pages 207-216, XP002057839 see the whole document ---	1-20
X	DRANOFF ET AL: "VACCINATION WITH IRRADIATED TUMOR CELLS ENGINEERED TO SECRETE MURINE GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR STIMULATES POTENT, SPECIFIC, AND LONG-LASTING ANTI-TUMOR IMMUNITY" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES,USA, vol. 90, 1993, pages 3539-3543, XP002057840 cited in the application see the whole document ---	1-20
A	GOLUMBEK ET AL: "CONTROLLED RELEASE, BIODEGRADABLE CYTOKINE DEPOTS: A NEW APPROACH IN CANCER VACCINE DESIGN" CANCER RESEARCH, vol. 53, 1993, pages 5841-5844, XP002057841 see page 5841 see abstract --- -/--	

1

INTERNATIONAL SEARCH REPORT

Inte. .onal Application No
PCT/US 97/18455

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	THOMPSON ET AL: "SYSTEMIC AND LOCAL PARACRINE CYTOKINE THERAPIES USING TRANSDUCED TUMOR CELLS ARE SYNERGISTIC IN TREATING INTRACRANIAL TUMORS" JOURNAL OF IMMUNOTHERAPY, vol. 19, no. 6, November 1996, pages 405-413, XP002057842 see the whole document -----	1-20

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 97/18455

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Remark : Although claims 9-20 are directed to a method of treatment of the human/animal body , the search has been carried out and based on the alleged effects of the compound/composition.